



Identification of the multivalent PDZ protein PDZK1 as a binding partner of sodium-coupled monocarboxylate transporter SMCT1 (*SLC5A8*) and SMCT2 (*SLC5A12*)

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Abstract

Sodium-coupled monocarboxylate transporters SMCT1 (*SLC5A8*) and SMCT2 (*SLC5A12*) mediate the high- and low-affinity transport of lactate in the kidney, but their regulatory mechanism is still unknown. Since these two transporters have the PDZ-motif at their C-terminus, the function of SMCTs may be modulated by a protein–protein interaction. To investigate the binding partner(s) of SMCTs in the kidney, we performed yeast two-hybrid (Y2H) screenings of a human kidney cDNA library with the C-terminus of SMCT1 (SMCT1-CT) and SMCT2 (SMCT2-CT) as bait. PDZK1 was identified as a partner for SMCTs. PDZK1 coexpression in SMCT1-expressing HEK293 cells enhanced their nicotinate transport activity. PDZK1, SMCT1, and URAT1 in vitro assembled into a single tri-molecular complex and their colocalization was confirmed in the renal proximal tubule in vivo by immunohistochemistry. These results indicate that the SMCT1-PDZK1 interaction thus plays an important role in both lactate handling as well as urate reabsorption in the human kidney.

Keywords Monocarboxylates · Monocarboxylate transporter · SMCT · Lactate · PDZ · PDZK1

Abbreviations

URAT1 Urate transporter 1
SLC Solute carrier
SMCTs Sodium-coupled monocarboxylate transporter
PDZ PSD-95, DglA, and ZO-1

Introduction

Lactate, one of the endogenous anionic metabolites, is freely filtered and extensively reabsorbed in the nephrons, similar to glucose and amino acids, to prevent loss of this valuable

metabolite from the body [1]. Several classical studies indicated that lactate uptake in the brush border membrane is performed by the sodium-monocarboxylate transport system [2]. Studies using isolated brush border membrane vesicles suggested the heterogeneity of the sodium-monocarboxylate transport system: a low-affinity, high-capacity system is present in the pars convoluta (S1 segment) of the proximal tubule and a high-affinity, low-capacity system is present in the pars recta (S3 segment) of the proximal tubule [3]. Recently, cDNAs encoding the human sodium-coupled monocarboxylate transporters (SMCTs) have been successively cloned [4–9].

SMCT1 (*SLC5A8*), the high-affinity lactate transport system, transports lactate, pyruvate, butyrate, propionate, acetate and nicotinate in a sodium-dependent manner [4–7]. SMCT2 (*SLC5A12*), the low-affinity lactate transport system, mediates the transport of several monocarboxylates in a sodium-dependent manner [8, 9]. Immunofluorescence study of mouse kidney revealed that Smct2 was broadly expressed throughout the proximal tubule (from S1 to S3 segments) while Smct1 was predominantly expressed in the late proximal tubule (S3 segment) [9]. These two transporters are thought to be responsible for renal reabsorption of lactate because the urinary excretion of lactate is markedly

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increased in a mouse model (*c/ebpδ* null mice) which lacks the expression of both transporters in the kidney [10].

In the same paper, they also reported that urinary excretion of urate is significantly elevated in *c/ebpδ*^{-/-} mice even though the expression of URAT1, the transporter responsible for apical membrane uptake of urate in the renal proximal tubule [11], is not altered [10]. Monocarboxylates that show the highest affinity for URAT1, such as nicotinate, pyrazinoate, followed by lactate, β-hydroxybutyrate and acetoacetate, are substrates for SMCT1 and SMCT2 [4–9]. An outwardly directed gradient of these monocarboxylates created by the Na⁺-coupled uptake by SMCT1 and SMCT2 may drive URAT1-mediated urate transport [12]. Therefore, the findings with *c/ebpδ* null mice may provide *in vivo* evidence for the functional coupling between lactate reabsorption and urate reabsorption in the kidney [10].

Although the transport properties and characteristics of substrate recognition for SMCTs have been documented recently, there is less information available on the regulation of SMCT function besides an epigenetic mechanism [13]. The expression of SMCT1 is down-regulated in a variety of tumors, including colonic tumors by the hypermethylation of CpG islands present in exon 1 of *SLC5A8* [14]. However, the modulation of the function of SMCTs by other mechanisms such as protein–protein interaction still remains unclear.

In recent years, several PDZ domain proteins, such as NHERF1/EBP50, NHERF2/E3KARP, and PDZK1, have been identified in the kidney and thus have been suggested to be involved in the stabilization, targeting, and regulation of their binding partner [15–18]. The PDZ (PSD-95, DglA, and ZO-1)-binding domains have been identified in various proteins and they are considered to be modular protein–protein recognition domains that play a role in protein targeting and protein complex assembly [19–21]. This domain binds to proteins containing the tripeptide motif (S/T)–X–Ø (X = any residue and Ø = a hydrophobic residue) at their C-termini [21]. SMCT1 and SMCT2 are localized at the apical membrane and have C-terminal amino acid sequences that match the PDZ-binding motif (T–R–L for SMCT1, T–H–F for SMCT2), in a manner similar to that of other apical organic

anion transporters, such as MRP2/4, NPT1, Oatp1, Oat-k1/k2; thus indicating that SMCT1 and SMCT2 most likely bind to certain PDZ domain proteins [22]. We previously identified that the urate/anion exchanger URAT1, which has a PDZ motif at its C-terminus (T–Q–F), interacts with PDZK1 [23]. Interestingly, both URAT1 and SMCTs are expressed at the apical membrane of renal proximal tubules and the interplay of these transporters is considered to be important in reabsorption of urate [12]. It is likely that these transporters bind to either the same or other PDZ domain protein(s) via its PDZ-motif. In this study, we performed yeast two-hybrid screening against a human kidney cDNA library using SMCT1-CT and SMCT2-CT as bait and identified PDZK1 as a physiological binding partner of SMCT1.

Materials and methods

Materials

[³H]nicotinic acid (55–59 Ci/mmol) was obtained from Moravsek (Brea, CA, USA). Fetal bovine serum and trypsin came from Invitrogen (Carlsbad, CA, USA). Human SMCT1 and SMCT2 cDNAs were gifts from Dr. Vadivel Ganapathy (Medical College of Georgia, Augusta, GA, USA). HA-tagged mouse *Urat1* transgenic mice were provided by Dr. Yoshikatsu Kanai (Osaka University, Osaka, Japan).

Plasmid construction

The C-terminal fragments of wild-type human SMCT1 and SMCT2 cDNAs and three mutants (designated d3, T608A and L610A for SMCT1, T616A and F618A for SMCT2) were generated by PCR using specific primers (Table 1) and cloned into the Eco RI and Xho I sites of pEG202 (bait) and pGEX-6P-1 (Amersham Biosciences) to construct SMCT1-CTwt, SMCT1-CTd3, SMCT1-T608A, SMCT1-L610A, SMCT2-CTwt, SMCT2-CTd3, SMCT2-T616A, and SMCT2-F618A. The full-length coding sequences of human SMCT1 wild type, human SMCT2 wild type, was inserted

Table 1 Primers used in the bait vector construction

Construct	Sense primer	Antisense primer
SMCT1-CTwt	5'-CGGAATTCACAGGAGGAAGAAAACAG-3'	5'-CATCTCGAGTCACAACAGGAGTCCCATTG-3'
SMCT1-CTd3	5'-CGGAATTCACAGGAGGAAGAAAACAG-3'	5'-CATCTCGAGCCCATTGCTCTTGCCACTCTCATG-3'
SMCT1-T608A	5'-CGGAATTCACAGGAGGAAGAAAACAG-3'	5'-CATCTCGAGTCACAACAGAGCCCCATTGCTCTTG-3'
SMCT1-L610A	5'-CGGAATTCACAGGAGGAAGAAAACAG-3'	5'-CATCTCGAGTCACGCCAGAGTCCCATTGCTCTTG-3'
SMCT2-CTwt	5'-GCGAATTCCTGGCTTTTGGTCTAAGAAG-3'	5'-CTCCTCGAGTTAGAAATGGGTAGTCTC-3'
SMCT2-d3	5'-GCGAATTCCTGGCTTTTGGTCTAAGAAG-3'	5'-CTCCTCGAGTTAAGTCTCAAATGCCATATTG-3'
SMCT2-T616A	5'-GCGAATTCCTGGCTTTTGGTCTAAGAAG-3'	5'-CTCTTCGAGTTAGAAATGGGCATGCTCAAATGC-3'
SMCT2-T618A	5'-GCGAATTCCTGGCTTTTGGTCTAAGAAG-3'	5'-CTCTTCGAGTTAGGCATGGGTATGCTCAAATGC-3'

into the mammalian expression plasmid pcDNA3.1 (Invitrogen) for functional analysis. The pcDNA3.1 vector containing the full-length human PDZK1 (hPDZK1) and preys (pJG4-5 and pMAL-c2x) containing single PDZ domains of hPDZK1 were prepared as described previously [24]. The C-terminal fragment used in the previous study was subcloned into the pMAL-c2x vector (New England Biolabs) to produce Maltose binding protein (MBP)-fused URAT1 C-terminal proteins.

Yeast two-hybrid assay

A human kidney cDNA library was constructed as described previously [23]. SMCT1 C-terminal bait corresponding to its last 49 amino acids and SMCT2 C-terminal bait corresponding to its last 55 amino acids were used to screen the human kidney cDNA library with the LexA-based *GFP* two-hybrid system (Grow'n'Glow system; MoBiTec).

In vitro binding assay

SMCT1-CT and SMCT2-CT were inserted into the pGEX-6P-1 plasmid (Amersham Biosciences) for GST fusion protein production in bacteria as reported previously [25]. Briefly, the bacterial pellet was resuspended in sonication buffer (phosphate buffer pH 7.4 containing protease inhibitors (Roche) and sonicated for 4 min. Triton X-100 (1% final) was added to the mixture and incubated for 30 min on ice, which was then centrifuged for 5 min at 15,000 rpm at 4 °C. The supernatant was used for the protein binding assay. MBP-fused proteins consisting of URAT1 were also purified as described above. In vitro translation was performed from a plasmid carrying the full-length PDZK1 with the TNT T7 Quick for PCR DNA system (Promega) in the presence of Transcend Biotinylated tRNA (Promega), as described elsewhere [23]. Glutathione Sepharose™ 4B resin beads (GE Healthcare) were washed 5 times with ice cold 1% Triton X-100 in 1 × PBS prior to the application of GST-fused SMCT1 or SMCT2 fusion protein. Twenty-five microliters of the GST-fused SMCT1 or SMCT2 fusion protein lysates was incubated with the affinity resin for 2 h at 4 °C. Resin was washed again 5 times with 1% Triton X-100 in 1 × PBS. Twenty microliters of freshly prepared in vitro translated PDZK1 was added to the resin and incubated overnight at 4 °C. The resin was washed 3 times with 1% Triton X-100 in 1 × PBS and once with ice cold PBS and aspirated to remove excess solution. The resin bound with protein complexes was resuspended in 15 µl 2X Laemmli buffer, heated for 10 min at 60 °C and electrophoresed in 10% SDS-PAGE gels; the fractionated proteins were blotted onto polyvinylidene difluoride membranes. The precipitated proteins were detected with the Transcend Chemiluminescent Translation Detection System (Promega).

For trimolecular protein complex pull down, GST-fused SMCT1 or SMCT2 C terminus bound Glutathione Sepharose affinity resin was incubated together with 20 µl in vitro translated full length PDZK1 and 1 µl MBP fused URAT1-CT protein lysate, overnight at 4 °C. The protein complexes were eluted similarly as described above. MBP fusion proteins were immunoblotted by anti-MBP antiserum (Santa Cruz) and detected using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare).

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (Invitrogen) at 37 °C in 5% CO₂. Transient transfection with Lipofectamine 2000 (Invitrogen, Gaithersburg, MD) was performed according to the manufacturer's recommendations.

Nicotinate transport activity assay

HEK293 cells were plated on 24-well culture plates at a density of 2×10^5 cells/well 24 h prior to transfection, and they were transfected as described above. After 36 h, the culture medium was removed, and the cells were washed 3 times and incubated in serum-free Hank's solution [containing in mM: 125 NaCl, 5.6 glucose, 4.8 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 1.3 CaCl₂·2H₂O, 25 HEPES (pH 6.0)] for 10 min. The uptake study was started by adding 500 µl of solution containing 30 µM [³H]nicotinate to the plate. After 2 min, the cells were washed twice in an ice-cold solution, and lysed in 0.1 N NaOH for 20 min for scintillation counting.

Immunohistochemical analysis

Frozen kidney tissue sections of HA-tagged Urat1 transgenic mice were washed 3 times with phosphate buffer (CaCl₂ 0.1 mM, MgCl₂ 1 mM). The tissue section was then blocked for 1 h at room temperature with blocking buffer solution (16.7% fetal bovine serum (FBS), 0.45 M NaCl, 1 × PBS, 0.003% Triton X-100). Primary antibodies specific for mouse Smct1, raised in goat (Santa Cruz) and anti-HA antibodies raised in rat (Roche) were diluted in the blocking buffer and incubated overnight at 4 °C. The tissue sections were washed 3 times with permeabilization buffer (1 × PBS, 0.3% Triton X-100, 0.1% BSA). Fluorophore-conjugated secondary antibodies, Alexa Fluor donkey anti-goat 488 (Invitrogen, green fluorescence for Smct1) and Alexa Fluor donkey anti-rat 594 (Invitrogen, red fluorescence for HA-tagged Urat1), DAPI (1 mg/ml, for nucleus) were diluted in the blocking buffer solution and incubated for 1 h

at room temperature. The tissue section was again washed 3 times in permeabilization buffer and once with PBS. The tissue sections were mounted and visualized with an Olympus Fluoview Confocal microscope.

Statistical analysis

Uptake experiments were conducted 3 times, and each uptake experiment was performed in triplicate. Values are presented as the mean \pm standard error. Statistical significance was determined by Student's *t*-test.

Results

Identification of PDZK1 by yeast two-hybrid library screening

In an attempt to identify SMCT1- and SMCT2-interacting protein(s), we performed yeast two-hybrid screening against a cDNA library constructed from the human adult kidney using the SMCT1-CT and SMCT2-CT as bait. From the 3.7×10^6 transformants screened by SMCT1-CT bait, we obtained 22 positive clones. Thirteen of these clones had a sequence identical to a portion of the human PDZK1 gene [26]. From the 1.2×10^7 transformants screened by SMCT2-CT bait, we obtained 34 positive clones. Eight of these clones had a sequence identical to a portion of the human PDZK1 gene. We could not detect any interactions between SMCT1-CT and SMCT2-CT and other apical PDZ proteins such as NHERF1/EBP50 [27, 28], NHERF2/E3KARP [29] or IKEPP [30] (data not shown).

C-terminal PDZ motif of SMCT1 and SMCT2 is necessary for PDZK1 interaction

To identify the regions of SMCT1 and SMCT2 that interact with PDZK1, we constructed three mutant baits: baits (SMCT1-CTd3 and SMCT2-CTd3) that lacked the last three residues of SMCT1 and SMCT2, which have been shown to play a crucial role in PDZ domain recognition. In two other baits (T608A/T616A and L610A/F618A), the extreme C-terminal leucine or phenylalanine (0 position) or threonine (−2 position) of SMCT1 and SMCT2 was replaced by alanine, which was expected to abolish the PDZ interactions [31]. These three baits did not interact with PDZK1 both in SMCT1 (Fig. 1a) and SMCT2 (Fig. 1b). Therefore, the binding through SMCT1-CT and SMCT2-CT suggests that the PDZ motif of SMCT1 and SMCT2 is the site of interaction with PDZK1.

A

	C terminal	LEU2	GFP
hSMCT1-CTwt	S N G T R L*	+	+
hSMCT1-CTd3	S N G*	−	−
hSMCT1-T608A	S N G A R L*	−	−
hSMCT1-L610A	S N G T R A*	−	−

B

	C terminal	LEU2	GFP
hSMCT2-CTwt	F E T T H F*	+	+
hSMCT2-CTd3	F E T*	−	−
hSMCT2-T616A	F E T A H F*	−	−
hSMCT2-F618A	F E T T H A*	−	−

Fig. 1 Specificity of PDZK1 interaction with C-termini of SMCT1 and SMCT2 in yeast two-hybrid system. **a** PDZK1 specifically interacted with the wild-type SMCT1 C-terminus but not with the C-terminal mutants T608A, L610A and d608-610 (d3) of SMCT1. **b** PDZK1 specifically interacted with the wild-type SMCT2 C-terminus but not with the C-terminal mutants T616A, F618A and d616-618 (d3) of SMCT2. pJG4-5 with PDZK1 cDNA expression cassette is under the control of the GAL1 promoter, such that library proteins are expressed in the presence of galactose (Gal) but not glucose (Glu). The system used for the yeast two-hybrid screen includes the reporter genes LEU2 and GFP, which replace the commonly used classical *lacZ* gene and allow a fast and easy detection of positive clones with long-wave UV. The results from the growth assay and GFP fluorescence are indicated on the right

Interaction of PDZK1 individual PDZ domains with SMCT1-CT and SMCT2-CT

PDZK1 possesses four PDZ domains that facilitate the assembly of protein complexes when binding target proteins via their C-terminal PDZ motifs. To determine the possible interactions of SMCT1-CT and SMCT2-CT with the individual PDZ domains of PDZK1, we produced prey vectors containing one of the individual PDZ domains [PDZ domain 1 (PDZ1), PDZ2, PDZ3, and PDZ4] from PDZK1. The interaction with SMCT1-CT was observed for PDZ1 and PDZ3, but not for PDZ2 or PDZ4 of PDZK1 (Fig. 2a), and the interaction with SMCT2-CT was observed for PDZ1, PDZ2 and PDZ4, but not for PDZ3 of PDZK1 (Fig. 2b).

In vitro binding of SMCT1 and SMCT2 and PDZK1

We used a GST pull-down assay to confirm the ability of SMCT1-CT and SMCT2-CT to bind to PDZK1 in vitro and validate the protein–protein interaction (Fig. 3). GST fusion proteins bearing the wild-type C-terminus (SMCT1-CTwt) or C-terminal mutants (SMCT1-CTd3, T608A and L610A) of SMCT1 were used to pull down in vitro translated full-length PDZK1 (PDZK1-FL). The data showed the same interaction specificity for PDZK1 and SMCT1 as exhibited in the yeast two-hybrid assay (Fig. 1a). As expected, the

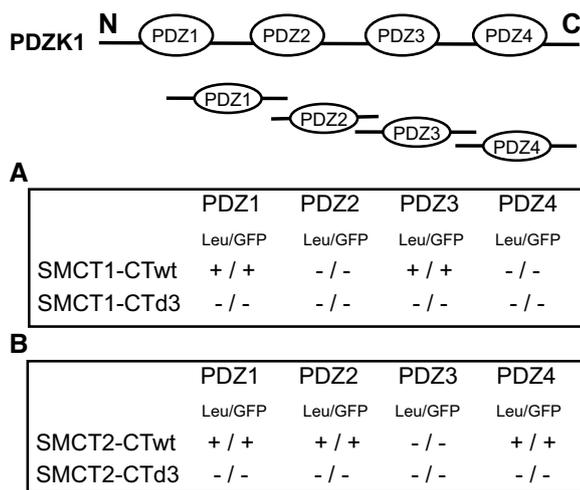


Fig. 2 Interaction of single PDZ domain of PDZK1 with C-termini of SMCT1 and SMCT2 in yeast two-hybrid system. **a** The wild-type SMCT1 C-terminus bait interacts with prey containing either the first or third PDZ domains of PDZK1 (PDZ1, PDZ3). The specificity of the prey containing a single PDZ domain of PDZK1 for the SMCT1 bait was confirmed by the absence of growth associated with the PEPT2 d3 mutant baits. **b** The wild-type SMCT2 C-terminus bait interacts with prey containing either the first, second or fourth PDZ domains of PDZK1 (PDZ1, PDZ2, PDZ4). The specificity of the prey containing a single PDZ domain of PDZK1 for the SMCT2 bait was also confirmed by the absence of growth associated with the PEPT2 d3 mutant baits. *Stop codon, Δ alanine mutation

binding of PDZK1 to SMCT1 was completely abolished when the C-terminal PDZ motif was removed (SMCT1-CTd3) or mutated (SMCT1-CT-T608A or SMCT1-CT-L610A) (Fig. 3a). GST fusion proteins bearing the wild-type C-terminus (SMCT2-CTwt) or C-terminal mutants (SMCT2-CTd3, T616A and F618A) of SMCT2 were used to pull down in vitro translated full-length PDZK1 (PDZK1-FL). The data showed the same interaction specificity for PDZK1 and SMCT2 as exhibited in the yeast two-hybrid assay (Fig. 1b). As expected, the binding of PDZK1 to SMCT2 was completely abolished when the C-terminal PDZ motif was removed (SMCT2-CTd3) or mutated (SMCT2-CT-T616A or SMCT2-CT-F618A) (Fig. 3b).

SMCT1 transport activity increases in presence of PDZK1

To determine whether SMCTs and PDZK1 interaction has any functional implications, transport activity of SMCTs was measured in the presence and absence of PDZK1 in a mammalian cell expression system. We transfected HEK293 cells with the pcDNA3.1(+) plasmid containing full-length SMCT1 (HEK-SMCT1-wt), full-length SMCT2 (HEK-SMCT2-wt), or without an insert (HEK-mock). After 2 min incubation, we demonstrated that [³H]nicotinate uptake via HEK-SMCT1 was approximately 1.8-fold higher than that

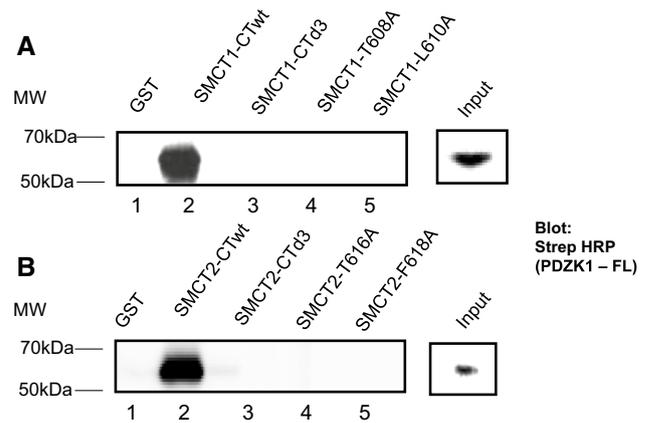


Fig. 3 Interaction of PDZK1 with SMCT1 and SMCT2. **a** Full-length PDZK1 PCR product was in vitro-translated in the presence of Transcend Biotinylated Lysine tRNA (Promega). The in vitro translation products were incubated with GST alone (lane 1), GST-SMCT1-CTwt (lane 2), GST-SMCT1-CTd3 (lane 3), GST-SMCT1-CT-T608A (lane 4), or GST-SMCT1-CT-L610A (lane 5) in a Glutathione Sepharose 4B affinity resin (GE Healthcare). The pull-down products were analyzed by SDS-PAGE. The input corresponds to the crude in vitro translation reaction. Positions of molecular mass standards are indicated on the left. GST fused to SMCT1 C-terminal wild type can coprecipitate PDZK1, confirming the specificity found in the yeast two-hybrid system. **b** Full-length PDZK1 PCR product was in vitro-translated in the presence of Transcend Biotinylated Lysine tRNA (Promega). The in vitro translation products were incubated with GST alone (lane 1), GST-SMCT2-CTwt (lane 2), GST-SMCT2-CTd3 (lane 3), GST-SMCT2-CT-T616A (lane 4), or GST-SMCT2-CT-F618A (lane 5) in a Glutathione Sepharose 4B affinity resin (GE Healthcare). The pull-down products were analyzed by SDS-PAGE. The input corresponds to the crude in vitro translation reaction. Positions of molecular mass standards are indicated on the left. GST fused to SMCT2 C-terminal wild type can coprecipitate PDZK1, confirming the specificity found in the yeast two-hybrid system

in HEK-mock and that in HEK-SMCT2 was approximately 1.3-fold higher than that in HEK-mock (Fig. 4). Nicotinate transport activities in HEK-SMCT1 significantly increased by PDZK1 coexpression (1.3-fold) and this effect was not observed when HEK-SMCT2 was coexpressed with PDZK1 (Fig. 4).

Formation of URAT1-PDZK1-SMCT1 multimolecular complexes in in vitro assay

To confirm the possibility that SMCT1 and SMCT2 are involved in renal urate reabsorption, we examined the coupling of URAT1 and SMCTs tethered by PDZK1 using an in vitro binding assay. As shown in Fig. 5, the SMCT1-PDZK1-URAT1 molecular complex was detected only when we incubated three components: GST-fused SMCT1-CT, MBP-fused URAT1-CT, and in vitro translated full-length PDZK1. The SMCT2-PDZK1-URAT1 molecular complex was also detected when we incubated GST-fused SMCT2-CT, MBP-fused URAT1-CT, and in vitro translated PDZK1

Fig. 4 Effect of PDZK1 on SMCT1 and SMCT2-mediated [3 H]nicotinate transport activity. Coexpression of SMCT1 and PDZK1 increased [3 H]nicotinate uptake (30 μ M) significantly over cells transfected with SMCT1 alone (*middle two columns*) while coexpression of SMCT2 and PDZK1 did not increase [3 H]nicotinate uptake (30 μ M) over cells transfected with SMCT2 alone (*right two columns*). $**p < 0.01$

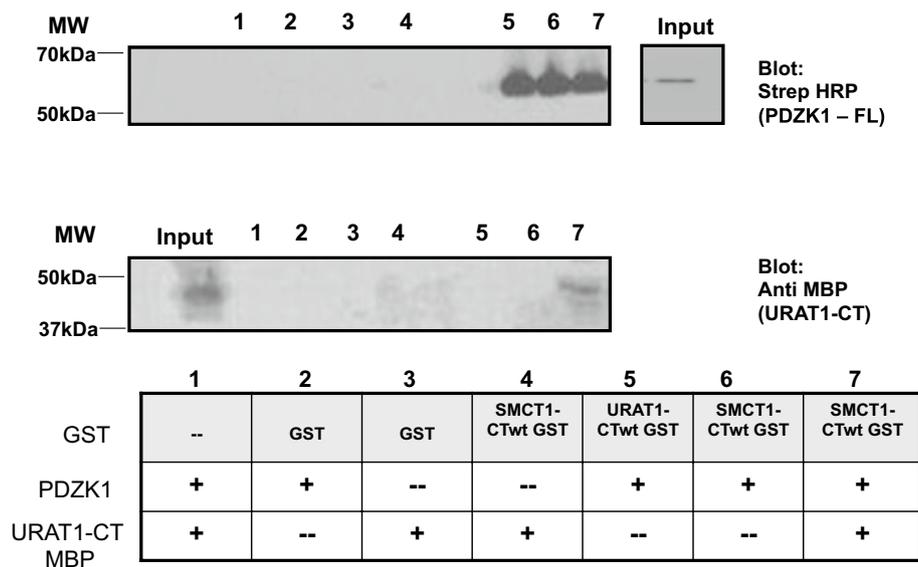
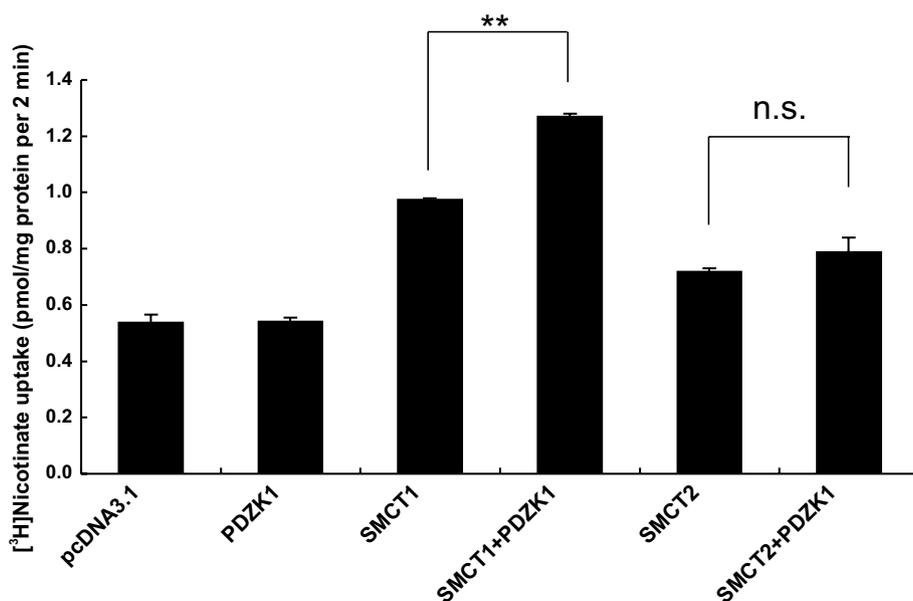


Fig. 5 Multimolecular complex formation including SMCT1-PDZK1-URAT1. GST-fused SMCT1-CTwt was incubated together with in vitro translated full length PDZK1 and MBP-fused URAT1-CTwt in a Glutathione Sepharose affinity column and were eluted and analyzed by SDS PAGE. The full length PDZK1 PCR product was in vitro-translated in the presence of Transcend biotinylated lysine tRNA (Promega). The in vitro translated products were incubated with MBP-URAT1-CTwt (*lane 1*), GST alone (*lane 2*), positive control: GST-URAT1-CTwt alone (*lane 5*), GST-SMCT1-CTwt (*lane*

6), GST-SMCT1-CTwt and MBP-URAT1-CTwt (*lane 7*), negative controls were GST alone and MBP-URAT1-CTwt without PDZK1 (*lane 3*) and GST-SMCT1-CTwt and MBP-URAT1-CTwt without PDZK1 (*lane 4*). MBP-fused URAT1 could only be precipitated in the presence of PDZK1 implicating the role of PDZK1 as a tether between SMCT1 and URAT1 in vitro. The input corresponds to the crude in vitro translation reaction and MBP-URAT1-CT lysate. The positions of molecular mass standards are shown on the *left*

(*Fig. 6*), but SMCT2-URAT1 interaction was observed without the addition of in vitro translated full-length PDZK1 (*Fig. 6*, *lane 4*), suggesting their direct interaction.

Colocalization of Smct1 and Urat1 in mouse kidney

In mouse, Smct1 and Urat1 as well as PDZK1 is present at the apical membrane of renal proximal tubules [9–11] and in humans, PDZK1 is reported to be expressed at the apical side of proximal tubular cells [23, 32]. To determine whether

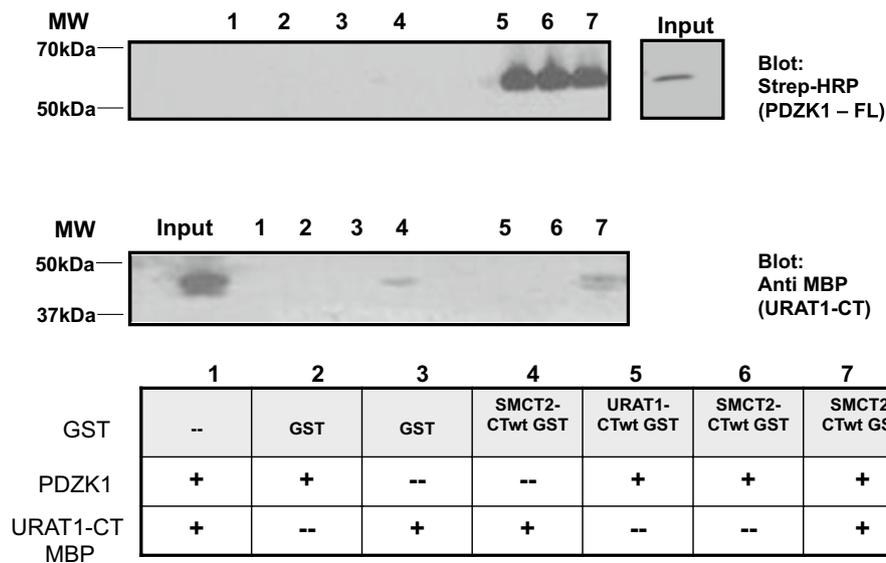


Fig. 6 Multimolecular complex formation including SMCT2-PDZK1-URAT1. GST-fused SMCT2-CTwt was incubated together with in vitro translated full length PDZK1 and MBP-fused URAT1-CTwt in a Glutathione Sepharose affinity column and were eluted and analyzed by SDS PAGE. The full length PDZK1 PCR product was in vitro translated in the presence of Transcend biotinylated lysine tRNA (Promega). The in vitro translated products were incubated with MBP-URAT1-CTwt (lane 1), GST alone (lane 2), positive control: GST-URAT1-CTwt alone (lane 5), GST-SMCT2-CTwt (lane 6), GST-SMCT2-CTwt and MBP-URAT1-CTwt (lane 7),

negative controls were GST alone and MBP-URAT1-CTwt without PDZK1 (lane 3) and GST-SMCT2-CTwt and MBP-URAT1-CTwt without PDZK1 (lane 4). GST-fused SMCT2-CTwt can coprecipitate PDZK1 along with MBP-fused URAT1-CTwt. MBP-URAT1-CTwt could also be precipitated with GST-SMCT2-CTwt in the absence of PDZK1, confirming that PDZK1 interacts with SMCT2-CT and may not be necessary for the interaction between URAT1 and SMCT1. The input corresponds to the crude in vitro translation reaction and MBP-URAT1-CT lysate. The positions of molecular mass standards are shown on the left

SMCT1 and URAT1 colocalize at the apical membrane of renal proximal tubules, we carried out immunostaining of the serial kidney sections from HA-tagged mouse *Urat1* transgenic mice using anti-Smct1 antibody. Consistent with previous reports, in the renal cortex, Smct1 immunoreactivities were detected at the apical side of proximal tubular cells (presumably S3 segments) together with *Urat1* detected by anti-HA antibody (Fig. 7).

Discussion

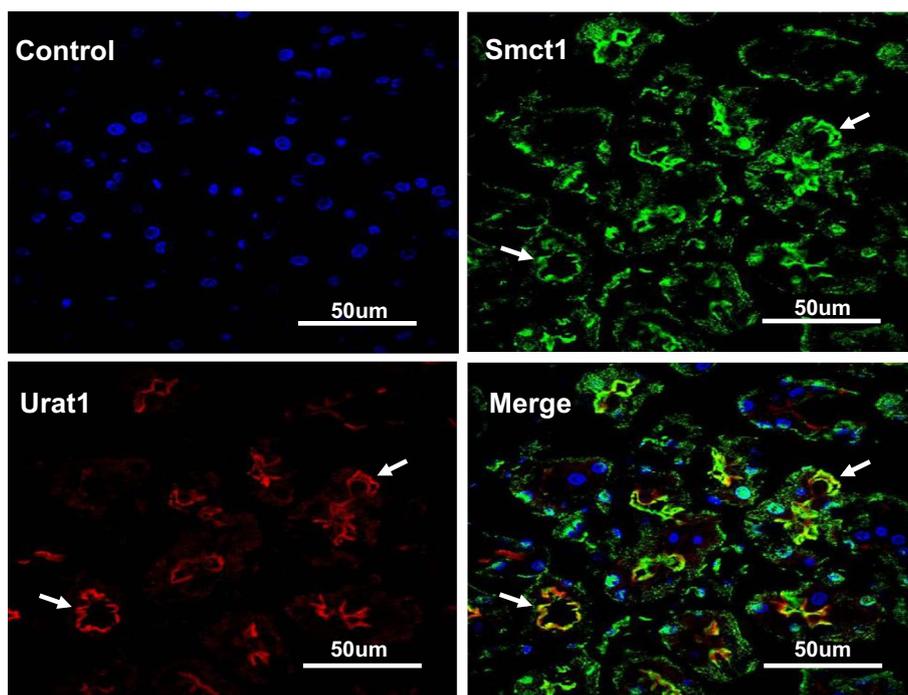
The sodium-coupled monocarboxylate transporters SMCT1 (*SLC5A8*) and SMCT2 (*SLC5A12*) mediate the high- and low-affinity transport of lactate in the kidney. In addition to the renal lactate metabolism, SMCTs may be involved in renal urate reabsorption by providing an outward-directed lactate gradient for urate influx via apical urate/anion exchanger URAT1. Therefore, SMCT1 and SMCT2 are presumed to contribute to the conservation of lactate as well as renal urate reabsorption. Although the transport properties and characteristics of substrate recognition for SMCTs have recently been documented, there is less information on SMCT regulation. To date, the yeast two-hybrid screens performed by Gisler et al., using baits containing single PDZ

domains derived from mouse *Pdzk1*, could not detect *Slc5a8* nor *Slc5a12* as candidates for PDZK1 binding partners [33]. To identify a physiological binding partner of SMCTs, we performed a yeast two-hybrid screening of a human kidney cDNA library using SMCT1-CT and SMCT2-CT as bait.

Significant PDZ interaction with both SMCT1 and SMCT2 was confirmed by several experiments. First, we could detect PDZK1 13 times from 22 positive clones in SMCT1-CT (59%) and 8 times from 34 positive clones in SMCT2-CT (23.5%) by library screening. These ratios are similar to the case of URAT1-PDZK1 interaction as previously reported (35.7%) [23]. Second, we could observe that SMCT mutants lacking the last 3 amino acids and those with alanine replacement lost the interaction with PDZK1 in yeast two-hybrid assays (Fig. 1) and in in vitro binding (Fig. 3). These results indicate that the interaction between SMCT1 and PDZK1 and that between SMCT2 and PDZK1 need PDZ interaction via their C-termini.

The interaction between SMCT1 and PDZK1 and that between SMCT2 and PDZK1 seem specific for each other, because the binding profiles for individual PDZ domains of PDZK1 were different. As shown in Fig. 2, SMCT1-CT interacted with PDZ1 and PDZ3 (Fig. 2a), while SMCT2-CT interacted with PDZ1, PDZ2, and PDZ4 (Fig. 2b). The binding site profile of SMCT2-CT to PDZK1 was identical

Fig. 7 Immunofluorescence analysis of Smct1 and Urat1 in the kidney sections from HA-tagged Urat1 transgenic mice. Immunofluorescences of Smct1 (green fluorescence) and Urat1 (red fluorescence) in kidney tissue sections of HA-tagged Urat1 transgenic mice were performed using their specific antibodies. Both Smct1 and Urat1 are expressed in the apical membrane of proximal tubular cells and were co-localized in the segments of proximal tubules. Arrow indicates the colocalization of Smct1 and Urat1 (color figure online)



to that of URAT1-CT [23], but that of SMCT1 has not been reported yet in the transporters that show the interaction with PDZK1.

Although both SMCT1 and SMCT2 interacted with PDZK1, we could only observe the effect of PDZK1 coexpression on the transport function of SMCT1, but not on SMCT2 (Fig. 4). This may be caused by their different expression efficiencies. As Gopal et al. reported, transport function of SMCT2 is low when they are expressed in HRPE cells [9]. If the transport rate of nicotinate by SMCT2 were at the same level as SMCT1, we may have found some differences in SMCT2 transport function in the presence of PDZK1.

Since URAT1 transports urate in exchange for intracellular organic anions such as lactate and nicotinate, uptake of such organic anions through SMCTs are thought to be important for the urate transport function of URAT1 [12]. As shown in Fig. 5, the SMCT1-PDZK1-URAT1 molecular complex was detected only when we incubated three components such as GST-fused SMCT1-CT, MBP-fused URAT1-CT, and in vitro translated full-length PDZK1. This result indicates that the functional coupling of URAT1 and SMCT1 is possible via PDZK1. In contrast, although the SMCT2-PDZK1-URAT1 molecular complex was also detected when we incubated GST-fused SMCT2-CT, MBP-fused URAT1-CT, and in vitro translated PDZK1, the SMCT2-URAT1 interaction was present without the addition of in vitro translated full-length PDZK1 (Fig. 6, lane 4). This result indicates that the interaction of SMCT2 and URAT1 can exist without PDZK1. Questions such as

whether this direct interaction occurs in vivo, and if it does, whether this interaction bears any physiological significance in renal urate handling remain to be solved. Regardless, we propose the existence of at least one “urate transportsome” consisting of URAT1, SMCT1, and PDZK1 in the kidney proximal tubule, which enables efficient urate reabsorption through functional coupling of apical anion transporters via a scaffold protein, PDZK1 [34].

Conclusion

We found that PDZK1 is a binding partner for both SMCT1 and SMCT2 and this protein may regulate renal lactate handling. In addition, we confirmed SMCT1-PDZK1-URAT1 trimolecular complex formation in vitro, suggesting that the SMCT1-PDZK1 interaction also plays an important role in urate reabsorption in the human kidney.

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Author contributions NA designed the study. SS and NA performed the Y2H. NA, KN and SM checked and confirmed the experimental

materials and data used in this study. SS and XH performed the transport assay. SS, TK and TF performed the biochemical and immunohistochemical analysis. SS, NA and HS wrote the manuscript. All authors discussed the results and commented on the manuscript.

Compliance with ethical standards

Conflict of interest All authors of this manuscript declare that we have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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