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PDZK1 Directly Regulates the Function of Organic Cation/Carnitine Transporter OCTN2

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ABSTRACT

Urinary excretion of cationic xenobiotics is believed to be mediated by organic cation transporter (OCT and OCTN) families expressed on both basolateral and brush-border membranes of renal tubules, although the molecular mechanisms for targeting of these transporters to each membrane are poorly understood. Here, to examine the regulatory mechanisms for cell-surface expression and function of these transporters, we evaluated the interaction of these transporters with several PDZ proteins. A pull-down study using recombinant C-terminal proteins of OCTs and OCTNs identified a specific interaction of apical transporters OCTN1 and OCTN2, but not basolateral transporters OCT1 and OCT2, with PDZK1, intestinal and kidney-enriched PDZ protein, and Na^+/H^+ exchanger regulatory factor 2 (also called E3KARP, SIP-1, or TKA-1). Both yeast two-hybrid and pull-down studies suggested a requirement of the last four amino acids in OCTN1 and OCTN2 for the interaction. The interaction of PDZK1 with the C terminus of OCTN2 was also confirmed in a pull-down study using kidney brush-border membrane vesicles. Immunohistochemical analysis revealed that both PDZK1 and OCTN2 are colocalized in brush-border membranes of the kidney. Finally, double transfection of OCTN2 with PDZK1 stimulated the uptake by OCTN2 of its endogenous substrate carnitine, and this increase could be accounted for by the 6-fold increase in transport capacity. Such an increase was not observed for OCTN2 with deletion of the last four amino acids. Biotinylation study of surface proteins revealed minimal effect of PDZK1 on cell-surface expression of OCTN2. The present findings are the first to identify PDZK1 as a functional regulator of OCTN2 through direct interaction with the C terminus.

Renal excretion of xenobiotics is believed to play a fundamental role in protecting the body against toxic compounds. This process includes glomerular filtration and secretion through both basolateral and apical surface membranes of renal tubular epithelial cells. The basolateral uptake of organic cations is membrane potential-dependent, whereas the apical membrane transport involves an H^+/cation exchanger system. Recent progress in molecular biological studies has revealed the existence of several organic cation transporter (OCT and OCTN) families that belong to the solute carrier (SLC) superfamily (Jonker and Schinkel, 2004; van Montfoort et al., 2003). Grundemann et al. (1994) first identified rat OCT1 (rOCT1) by means of an expression cloning strategy, followed by the cloning

of rOCT2 and rOCT3 (Okuda et al., 1996; Kekuda et al., 1998). rOCT1 and rOCT2 accept various organic cations, such as tetraethylammonium (TEA), 1-(methyl-d3)-4-phenylpyridinium, choline, and *N*-methylnicotinamide, as substrates (Grundemann et al., 1994; Busch et al., 1996; Nagel et al., 1997; Kekuda et al., 1998; Koepsell, 1998; Sweet and Pritchard, 1999; Budiman et al., 2000) and are expressed on basolateral membranes in kidney (Jonker and Schinkel, 2004). The OCT family was also cloned from humans (Gorboulev et al., 1997; Zhang et al., 1997; Wieland et al., 2000), and human OCT2 is expressed on the basolateral cell surface in kidney (Gorboulev et al., 1997). Recent findings in OCT1 and OCT2-null mice strongly suggests a predominant role of these transporters in basolateral uptake of organic cations in kidney (Jonker et al., 2003; Jonker and Schinkel, 2004).

Our group has previously cloned OCTN1 from fetal liver; this transporter showed approximately 30% homology with

ABBREVIATIONS: OCT, organic cation transporter; PDZ, postsynaptic density 95/disc-large/ZO-1; NHERF, Na^+/H^+ exchanger regulatory factor; IKEPP, intestinal and kidney-enriched PDZ protein; SLC, solute carrier; GST, glutathione *S*-transferase; TEA, tetraethylammonium; OAT, organic anion transporter; NHE3, Na^+/H^+ exchanger 3; MAP, membrane-associated protein; NPT, Na^+-dependent phosphate transporter; PCR, polymerase chain reaction; aa, amino acid; HEK, human embryonic kidney; BBMV, brush-border membrane vesicle; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein; LDL, low-density lipoprotein; r, rat.

the OCT and organic anion transporter (OAT) families (Tamai et al., 1997). Gene expression of OCTN1 is ubiquitous, including the kidney (Tamai et al., 1997). OCTN1 transports TEA in a pH-dependent but Na$^+$-independent manner. The kinetic evidence obtained from membrane vesicles expressing OCTN1 indicates both H$^+$/cation and cation/cation exchange activity (Tamai et al., 2004). The localization of OCTN1 on apical membranes in kidney also suggests the possible involvement of OCTN1 in the H$^+$/cation exchange system on kidney brush-border membranes (Yabuuchi et al., 1999; Tamai et al., 2004). The OCTN1 gene is possibly associated with rheumatoid arthritis (Tokuhiro et al., 2003). On the other hand, OCTN2, which has 76% homology with OCTN1, is a sodium-dependent high-affinity transporter for carnitine and is also expressed on renal apical membranes (Tamai et al., 1998, 2001; Wu et al., 1998; Ohashi et al., 1999). Deficiency of the OCTN2 gene causes systemic carnitine deficiency (Nezu et al., 1999), possibly because of reduced reabsorption of carnitine in kidney tubules. OCTN2 also accepts organic cations as substrates (Ohashi et al., 1999; Tamai et al., 2000). Renal excretion of TEA in juvenile visceral steatosis mice, which have a hereditary deficiency of the OCTN2 gene, is reduced, compared with normal mouse, indicating a role of OCTN2 in renal excretion of organic cations (Ohashi et al., 2001).

Thus, recent findings have suggested that both basolateral (OCT1 and OCT2) and apical (OCTN1 and OCTN2) transporters are cooperatively involved in renal excretion of organic cations. Therefore, to understand the factors that determine the unidirectional transport of cationic xenobiotics from the blood into urine, it is essential to clarify the molecular mechanisms involved in the targeting of these transporters to the appropriate surface of plasma membranes. At present, no information is available on the sorting machinery for organic cation transporters. One of the key issues regarding the apical expression of transmembrane proteins is their direct interaction with PDZ (PSD-95, Dlg, and ZO-1) domain-containing proteins, which are also expressed on apical membrane surfaces in kidney (Biber, 2001; Voltz et al., 2001). A number of pioneering studies have revealed four PDZ proteins, PDZK1, intestinal and kidney-enriched PDZ protein (IKEPP), Na$^+$/H$^+$ exchanger regulatory factor (NHERF) 1, and NHERF2, which are expressed in various organs, including kidney and intestines (Reczek et al., 1997; Yun et al., 1997; Wang et al., 2000; Gisler et al., 2001) and can directly interact with the SLC superfamily (Kocher et al., 1999; Gisler et al., 2001, 2003; Raghuram et al., 2001; Lohi et al., 2003). NHERF1 was first identified as a regulatory factor for Na$^+$/H$^+$ exchanger 3 (NHE3). NHERF1 is associated with actin microfilaments through interaction with ezrin-radixin-moesin proteins and is expressed on the apical surface of renal tubular epithelial cells. NHERF1 regulates the function and localization of NHE3 by protein kinase A-dependent phosphorylation (Volts et al., 2001) and is believed to be involved in the regulation of apical localization of NHE3 by parathyroid hormone (Mahon et al., 2003). NHERF2, which has 57% homology with NHERF2, is also expressed on apical membranes of the kidney and is believed to play a similar role in regulating NHE3 function (Yun et al., 1997; Wade et al., 2003). PDZK1 was first identified by yeast two-hybrid analysis as a protein which interacts with membrane-associated protein (MAP) 17 that is highly expressed in human carcinomas (Kocher et al., 1998). PDZK1 is also called NaPi-Cap1, because it also interacts with Na$^+$-dependent phosphate transporter (NPT) 2a (Hernando et al., 2002). Recent findings suggest the interaction of PDZK1 with several xenobiotic transporters, including NPT1, OCTN1, organic anion transporting polypeptide 5, and multidrug resistance-associated protein 2, although the physiological relevance of such interactions remains to be determined (Gisler et al., 2003). IKEPP is homologous to PDZK1 and is highly expressed in kidney and intestines (Scott et al., 2002). This protein also interacts with the C terminus of NPT2a and multidrug resistance-associated protein 2 (Gisler et al., 2001; Hegedus et al., 2003).

The unique structural features of PDZ proteins include the existence of multiple PDZ domains that can directly interact with each other. For example, both NHERF1 and NHERF2 have two PDZ domains and interact with each other. PDZK1 has four PDZ domains and interacts with NHERF1 (Gisler et al., 2003). These features, along with the apical localization of the molecules and direct interaction with transmembrane proteins, imply the existence of a protein network consisting of various types of cell-surface proteins. However, limited information is still available on protein interactions of the OCT and OCTN families and their physiological relevance. In addition, the regulatory mechanisms of these transporters have not yet been identified, despite their likely physiological importance. In the present study, we examined the protein interactions and functional regulation of these transporters as a first step in identifying the molecular mechanisms that control their expression and function.

Materials and Methods

Materials. Rabbit polyclonal antibody for OCTN2 was against a synthetic peptide corresponding to amino acid residues (CTRMQKDG-EESPTVLKSTAF) of mouse OCTN2 (Tamai et al., 2000). Monoclonal antibody 9E10 (c-myc) was purchased from Covance Inc. (Princeton, NJ), and monoclonal antibody against Na$^+$/K$^+$ ATPase was from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-His$_5$ antibody was purchased from BD Biosciences Clontech (Palo Alto, CA). Rat polyclonal antibody for PDZK1 was raised against a synthetic peptide corresponding to amino acid residues (STASHSSSNSEDTEM) of human PDZK1. L-[^{3}H]Carnitine (3108 GBq/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). The myc epitope sequence (MEEQKLISEEDL) was first introduced into the BamHI site of pcDNA3 (Invitrogen, Carlsbad, CA) plasmid. cDNA clones encoding PDZK1, NHERF1, and NHERF2 were obtained from Invitrogen, whereas cDNA for IKEPP was cloned by PCR amplification (Advantage2; BD Biosciencs Clontech) using cDNA obtained from Caco-2 cells as a template. cDNA fragments encoding single or multiple PDZ domains (listed below) were obtained by PCR amplification: aa 1 to 101 for PDZ1, aa 119 to 221 for PDZ2, aa 230 to 351 for PDZ3, aa 365 to 519 for PDZ4, aa 1 to 221 for PDZ1–2, aa 119 to 351 for PDZ2–3, and aa 230 to 519 for PDZ3–4. All of these cDNAs were subcloned into pGADT7 (BD Biosciences Clontech) and myc/pcDNA3 plasmids. cDNA fragments encoding the C-terminal 41, 41, 39, 44, and 48 amino acids of human OCT1, OCT2, OCT3, OCTN1, and OCTN2, respectively, were amplified by PCR and subcloned into both pGBKT7 (BD Biosciences Clontech) and pGEX6P-1 (Amersham Biosciences) plasmids between the EcoRI and SalI sites. The C-terminal four amino acids in OCT1 (PSGT) and OCT2 (IPLN) were replaced with those of OCTN1 (IATF) or OCTN2 (STAF) to obtain four chimeric peptides OCT1N1, OCT1N2, OCT2N1, and OCT2N2. The full sequences of all of the inserts were verified. Glutathione S-transferase (GST) fusion proteins and His$_6$-tagged PDZK1 were obtained from *Escherichia coli*

(BL-21 strain) transformed with pGEX6P-1 and pET30 constructs, respectively, according to the manufacturer's instructions.

Cell Culture. HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37°C and 5% CO_2. After 24-h cultivation, a cDNA construct was transiently transfected by adding 20 μg/15-cm dish of the plasmid DNA according to the calcium phosphate precipitation method (Tamai et al., 2000). HEK293 cells stably expressing myc construct of PDZK1 (HEK293/PDZK1 cells) were obtained previously (Kato et al., 2004).

Preparation of Brush-Border Membrane Vesicles from Mouse Kidney. Both kidneys were removed from C57BL/6 male mice (8 weeks) under ether anesthesia, and brush-border membrane vesicles (BBMVs) were prepared according to the previous report (Malathi et al., 1979). BBMVs were then suspended in the transport buffer containing 300 mM mannitol and 10 mM HEPES/Tris, pH 7.0, frozen in liquid nitrogen, and stored at −80°C until use.

Yeast Two-Hybrid Analysis. Yeast two-hybrid analysis was performed as described previously (Kato et al., 2004). In brief, yeast cells (AH109 strain) were cotransformed with pGBKT7(TRP) encoding GAL4bd fused to the C terminus of transporters and pGADT7(LEU2) vector encoding GAL4ad fused to different PDZ domain constructs. Cotransformed cells were further cultured on plates lacking leucine and tryptophan with or without histidine.

Pull-Down Experiments Using Recombinant GST Fusion Proteins. HEK293 cells at 48 h after transfection or HEK293/PDZK1 cells were washed twice with phosphate-buffered saline (PBS) and collected with a rubber policeman followed by centrifugation. The obtained pellet or mouse BBMVs (200 μg protein) were solubilized in radioimmunoprecipitation assay-Y buffer containing 1% Nonidet P-40, 75 mM NaCl, 50 mM Tris-HCl, pH 7.5, and protease inhibitors. The obtained lysate (500 μl) was then incubated with GST fusion protein (100 μg) and glutathione-Sepharose 4B at 4°C for 3 h, followed by washing three times with ice-cold PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-c-myc antibody. The membrane was then incubated with horseradish peroxidase-linked donkey anti-mouse IgG as the secondary antibody (Amersham Biosciences), and the protein was detected with the ECL Plus system. The amount of GST fusion protein added to the reaction mixture was checked with Ponceau red; staining was similar in all cases (data not shown). Pull-down studies using purified His$_6$-tagged PDZK1 were performed as described previously (Kato et al., 2004).

Transport Studies. cDNA encoding human OCTN2 or OCTN2 with the last four amino acids deleted was subcloned into pEYFP-C1 vector (BD Biosciences Clontech) and transiently transfected into HEK293 or HEK293/PDZK1 cells. At 48 h after transfection, cells were harvested and suspended in transport medium (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, and 25 mM HEPES, pH 7.4). The cell suspension and the transport medium containing L-[^{3}H]carnitine were mixed to initiate the transport reaction (Ohashi et al., 1999). At the designated times, 200-μl aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicon oil (SH550; Toray Dow Corning, Tokyo, Japan) and liquid paraffin (Wako Pure Chemicals, Osaka, Japan) with a density of 1.03 on top of 3 M KOH solution. After solubilization of each cell pellet in KOH, the cell lysate was neutralized with HCl. Then, the associated radioactivity was measured by using a liquid scintillation counter with Clearsol-1 (Nacalai Tesque, Kyoto, Japan) as a liquid scintillation fluid. Cellular protein content was determined using the Bradford method with a protein assay kit (Bio-Rad, Hercules, CA).

Biotinylation of Surface Proteins. At 48 h after transfection of YFP-OCTN2, the cells were harvested and washed three times with PBS. Cells (2.5 × 10^7 cells/ml) were then incubated at 4°C with 0.5 mg/ml sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL) for 1 h. After washing with PBS, the cells were again incubated with sulfo-NHS-

LC-Biotin in the same manner, followed by washing and solubilization with 20 mM phosphate buffer, pH 7.5, containing 1% Nonidet P-40, 75 mM NaCl, and protease inhibitors. Solubilized fraction was incubated at 4°C for 30 min with 50 μl of immobilized streptavidin (Roche Diagnostics, Indianapolis, IN) which was then washed three times with PBS containing 0.02% Tween 20 and subjected to Western blot analysis.

Western Blot Analysis of Membrane Fractions in HEK293 Cells. The harvested cell suspension was centrifuged and resuspended in a buffer consisting of 210 mM sucrose, 2 mM EGTA, 40 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 100 μM leupeptin, 2 μg/ml aprotinin, and 30 mM HEPES, pH 7.4, and homogenized by using a Polytron homogenizer (IKA Labortecnik, Staufen, Germany). Then, 800 μl of the homogenate was mixed with 750 μl of 1.17 M KCl solution containing 58.3 mM tetrasodium pyrophosphate and centrifuged at 230,000g for 75 min. The resultant pellet was suspended in a buffer consisting of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and centrifuged again at 230,000g. The obtained pellet was resuspended in the above buffer and ultrasonically dispersed. After the addition of 4% SDS solution, the solution was mixed and centrifuged at 15,000g. The resultant supernatant was used for Western blot analysis after the addition of a buffer consisting of 43.4 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, and the subsequent addition of urea. Each sample (10 μg of protein) was separated by 7.5% polyacrylamide gel electrophoresis, and proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane, Immobilon P (Millipore Corporation, Billerica, MA). The membrane was incubated in Tris-buffered saline (20 mM Tris-HCl, pH 7.5 and 137 mM NaCl) containing 0.1% Tween 20 and 10% skim milk for blocking, incubated with an anti-green fluorescent protein antibody (Roche Diagnostics) in the above buffer, and rinsed with Tris-buffered saline containing 0.1% Tween 20. It was then reacted with a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences), and signals were visualized using the enhanced chemiluminescence detection method with the ECL Plus Western blotting detection system (Amersham Biosciences). Quantitative analysis was done by densitometry using a light-capture apparatus (AE6961FC; Atto Bioscience, Tokyo, Japan).

Immunocytochemical Analysis. Frozen sections of mouse kidney were prepared as described previously (Tamai et al., 2004). The sections were first heated to 92°C in the RETRIEVE-ALL buffer (SIGENT Pathology Systems Inc., Dedham, MA). They were then incubated with a mixture of antibodies for 1 h and subsequently incubated with secondary antibodies (Alexa Fluoro 488 goat anti-mouse IgG conjugate, Alexa Fluoro 488 or 594 goat anti-rat IgG conjugate, and Alexa Fluoro 594 anti-rabbit IgG conjugate; Molecular Probes, Eugene, OR) for 30 min at room temperature. Finally, they were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to fix the sample. The specimens were examined with an Axiovert S 100 microscope and a confocal laser scanning fluorescence microscope (LSM 510; both from Carl Zeiss GmbH, Jena, Germany).

HEK293 cells were grown on microcover glasses (15 mm; thickness, 0.12–0.17 mm; Matsunami Glass Ind., Osaka, Japan) and transfected as described above. Two days after the transfection, cells were fixed with 3% formaldehyde in PBS, permeabilized with methanol for 5 min, and incubated with PBS containing 3% blocking agent (Amersham Biosciences) for 30 min at room temperature. Cells were incubated with anti-human PDZK1 antibody for 1 h at room temperature, washed with PBS, and then incubated with Alexa Fluor594 goat anti-rat IgG conjugate at a dilution of 1:200 in PBS containing 3% blocking agent. Finally, they were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories) to fix the sample. The specimens were examined with a confocal laser scanning fluorescence microscope (LSM 510; Carl Zeiss).

Estimation of Kinetic Parameters. Kinetic parameters (K_m and V_{max}) for transport activity of OCTN2 were estimated by fitting the data to the Michaelis-Menten equation with nonlinear least-

squares analysis using the MULTI program (http://www.pharm. kyoto-u.ac.jp/byoyaku/kinetics/download.html).

Results

Pull-Down Studies for Analysis of the Interaction between PDZ Proteins and Organic Cation Transporters. As a first step to identify regulatory proteins for cation transporters, pull-down studies were first performed for the four PDZ proteins (NHERF1, NHERF2, PDZK1, and IKEPP), which have previously been shown to interact with SLC superfamily proteins (Fig. 1). Lysates prepared from HEK293 cells expressing one of the four PDZ proteins were subjected to pull-down using GST fusion protein with the C terminus of each transporter. The C terminus of OCT3 interacted with IKEPP (65 kDa), whereas that of OCTN2 interacted with NHERF2 (~50 kDa), PDZK1 (~70 kDa), and IKEPP (Fig. 1), although the interaction with NHERF2 was very weak, and further studies are required to examine the physiological relevance of such a weak interaction. The previous findings (Gisler et al., 2003) demonstrating the interaction of OCTN1 with NHERF1 and PDZK1 were confirmed in this analysis, but, in addition, we found that the C terminus of OCTN1 can also bind to NHERF2 and IKEPP (Fig. 1). The C terminus of either OCT1 or OCT2 exhibited no interaction (Fig. 1). These observations were essentially similar to our previous results obtained by yeast two-hybrid screening, except that OCTN2 also bound to NHERF1 in the yeast analysis (Kato et al., 2004).

We next examined the structural requirement at the C terminus of OCTN1 and OCTN2 for the interaction. In pull-down studies, the C terminus of OCTN1 could bind with all four PDZ proteins, whereas the C terminus with the last four amino acids deleted (OCTN1Δ4) exhibited no interaction in each case (Fig. 2). The interaction between the C terminus of OCTN2 and NHERF2, PDZK1, or IKEPP was diminished when the last four amino acids of the C terminus were deleted (Fig. 3). In all cases

examined, nonspecific binding to GST alone was not detected (Figs. 2 and 3). It was noted that NHERF1, NHERF2, and PDZK1 exhibited at least two bands (Figs. 1–3), the identity of which has not yet been determined.

Yeast Two-Hybrid Analysis. Because the interaction of PDZK1 with OCTN1 and OCTN2 was most marked (Fig. 1), we next attempted to identify the PDZ domains in PDZK1 responsible for the interaction with these cation transporters. Both OCTN1 and OCTN2 interacted with full-length PDZK1 and a single PDZ domain PDZ1, PDZ2, or PDZ4 (Fig. 4a), the interaction with PDZ2 and PDZ4 being absent for OCTN1 and OCTN2 with the last four amino acids of the C terminus deleted (Fig. 4a). The interaction of OCTN1 and OCTN2 with PDZ1 was weak, but still present, when the last four amino acids were deleted (Fig. 4a). OCTN2 interacted with each combination of two sequential PDZ domains, such as PDZ1–2, PDZ2–3 and PDZ3–4 (Fig. 4b). These results suggest that OCTN2 can interact with PDZ2 and PDZ4.

Colocalization of PDZK1 and OCTN2 in Renal Proximal Tubules. Although PDZK1 and OCTN2 were reported to be expressed on the apical membranes of renal proximal tubules (Kocher et al., 1998; Tamai et al., 2001, 2004), there is no report to demonstrate their colocalization. To examine the possible interaction of PDZK1 and OCTN2 in kidney, pull-down study was performed using kidney BBMVs. The alkaline phosphatase activity of BBMVs prepared in the present study was 25.1 times higher than that of kidney homogenates. Pull-down study using GST fusion protein with the C terminus of OCTN1 and OCTN2 resulted in a single band of 70 kDa, which was immunoreactive with PDZK1 antibody (Fig. 5). This band was diminished when the last four amino acids were deleted in the GST fusion proteins (Fig. 5). In addition, neither GST fusion with the C terminus of OCT2 or OCT3 nor GST alone exhibited any immunoreactive band (Fig. 5).

Next, to examine the colocalization of PDZK1 and OCTN2, cryosections of mouse kidney were stained with polyclonal antibodies against PDZK1 and OCTN2. In each case, immunoreactive fluorescence was detected mainly on the luminal sides of proximal tubules, and this was in contrast with the antiluminal staining with Na^+-K^+ ATPase antibody (Fig. 6).
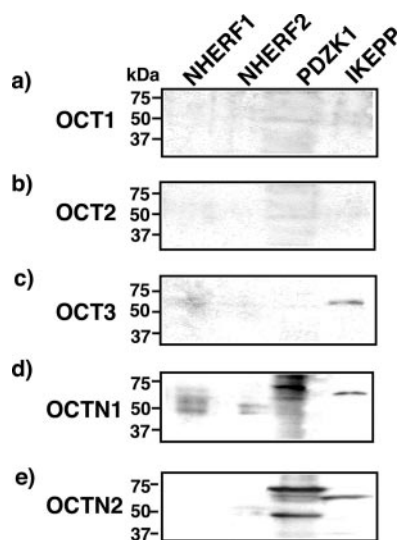
Fig. 1. Pull-down of various PDZ proteins with recombinant GST fusion protein having the C-terminal sequence of organic cation transporters. HEK293 cells transiently transfected with myc constructs containing full-length IKEPP, NHERF1, and NHERF2 or stably expressing myc-tagged PDZK1 were solubilized and subjected to pull-down analysis with GST fusion protein bearing the carboxyl terminus of OCT1 (a), OCT2 (b), OCT3 (c), OCTN1 (d), or OCTN2 (e). The precipitated (interacted) materials were then analyzed by Western blotting using c-myc (9E10) antibody.
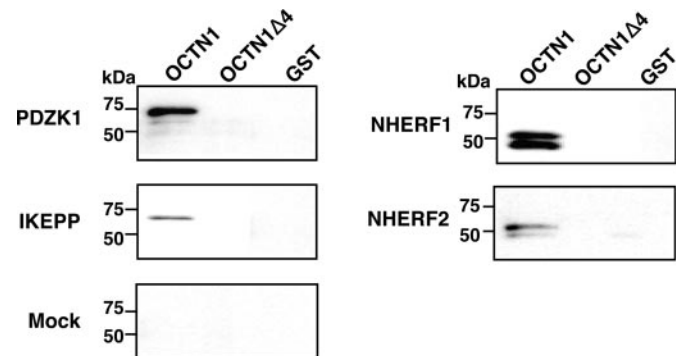

Fig. 2. The C-terminal four amino acids of OCTN1 are required for the interaction with PDZ proteins. HEK293 cells transiently transfected with myc constructs containing full-length IKEPP, NHERF1, and NHERF2 or stably expressing myc-tagged PDZK1 were solubilized and subjected to pull-down analysis with GST fusion protein bearing the carboxyl terminus of OCTN1, OCTN1 mutant with the carboxyl-terminal four amino acids truncated (OCTN1Δ4), or GST alone (GST). The precipitated (interacted) materials were then analyzed by Western blotting using c-myc (9E10) antibody. Mock indicates HEK293 transfected with vector (pcDNA3) only.

Immunostaining with PDZK1 antibody was almost superimposable on that with OCTN2 antibody (Fig. 6, g–i). The colocalization of PDZK1 and OCTN2 was also suggested by the immunostaining observed in confocal microscopy (Fig. 7). Thus, these results suggest a specific interaction between PDZK1 and OCNT2 in kidney.

Interaction of PDZK1 with OCT1 and OCT2 with Their C-terminal Four Amino Acids Replaced with Those of OCTN1 and OCTN2. To examine whether the C-terminal four amino acids of OCTN1 and OCTN2 are sufficient for interaction with PDZK1 or not, both yeast two-hybrid and pull-down studies were performed for C terminus of OCT1 and OCT2, the last four amino acids of which were
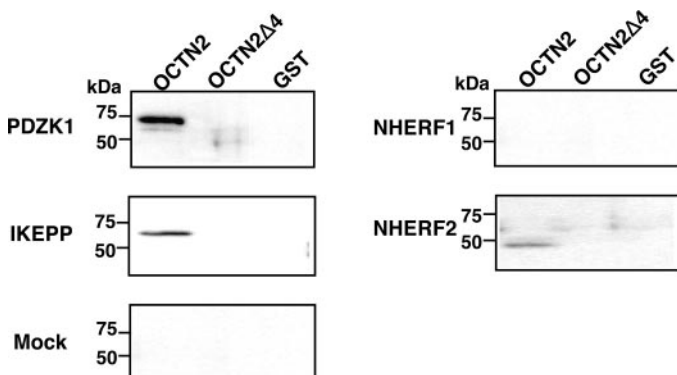
replaced with those of OCTN1 or OCTN2. The OCT-OCTN chimeric peptides thus obtained (OCT1N1, OCT1N2, OCT2N1, and OCT2N2) exhibited interaction with PDZK1 in yeast two-hybrid analysis, although the interaction of OCT2N1 with PDZK1 was very weak (Fig. 8a). Likewise, His$_6$-tagged PDZK1 was pulled down with GST fused with all the four OCT-OCTN chimeric peptides (Fig. 8b). The interaction was not so marked, compared with OCTN2, but it was more obvious than OCTN2Δ4. Thus, the C-terminal four amino acids in OCTN1 and OCTN2 seem to be sufficient for the interaction with PDZK1.

Functional Regulation of OCTN2 by PDZK1. OCTN2 plays an essential role in reabsorption of carnitine at renal brush-border membranes, and OCTN2 gene deficiency results in primary systemic carnitine deficiency (Koizumi et al., 1999; Nezu et al., 1999; Yokogawa et al., 1999). Direct interaction of OCTN2 with PDZK1 may suggest physiological regulation of OCTN2-mediated carnitine transport activity by PDZK1. To examine this hypothesis, we investigated the effect of coexpression of PDZK1 on the expression and carnitine transport activity of OCTN2 transiently expressed in HEK293 cells. An almost identical band intensity was observed between HEK293 and HEK293/PDZK1 cells at 84 kDa, which corresponded to YFP-OCTN2, in the Western blot analysis of membrane fractions (Fig. 9a) and biotinylated proteins (Fig. 9b). In HEK293 cells, transient transfection of YFP-OCTN2 results in higher uptake of carnitine than that found in mock-transfected cells (Fig. 9c). In HEK293/PDZK1 cells, higher uptake of carnitine was also observed after transient expression of YFP-OCTN2 compared with mock-transfected HEK293/PDZK1 cells, and such uptake by YFP-OCTN2 transfected in HEK293/PDZK1 cells was greater than that found in HEK293 cells (Fig. 9c). On the other hand, the uptake of carnitine by YFP-OCTN2Δ4, in which the last four amino acids of OCTN2 were deleted, was similar in each cell line (Fig. 9d). In the same study, confocal microscopic images of YFP-OCTN2 or YFP-OCTN2Δ4 in each cell line confirmed the expression of both YFP chimeric proteins on the cell surface (data not shown). We also transiently trans-
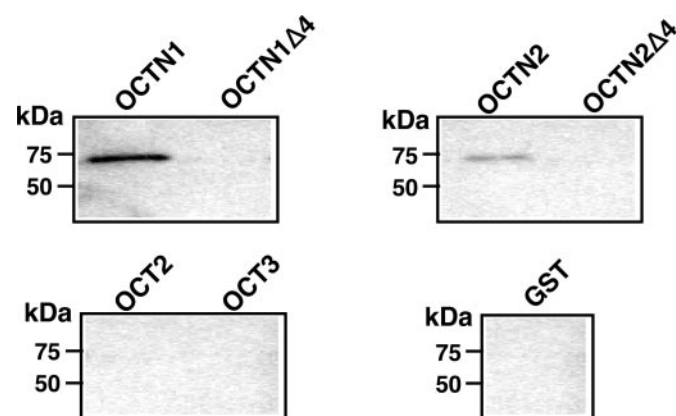

Fig. 3. The C-terminal four amino acids of OCTN2 are required for the interaction with PDZ proteins. HEK293 cells transiently transfected with myc constructs containing full-length IKEPP, NHERF1, and NHERF2 or stably expressing myc-tagged PDZK1 were solubilized and subjected to pull-down analysis with GST fusion protein bearing the carboxyl terminus of OCTN2, OCTN2 mutant with the carboxyl-terminal four amino acids truncated (OCTN2Δ4), or GST alone (GST). The precipitated (interacted) materials were then analyzed by Western blotting using c-myc (9E10) antibody. Mock indicates HEK293 transfected with vector (pcDNA3) only.
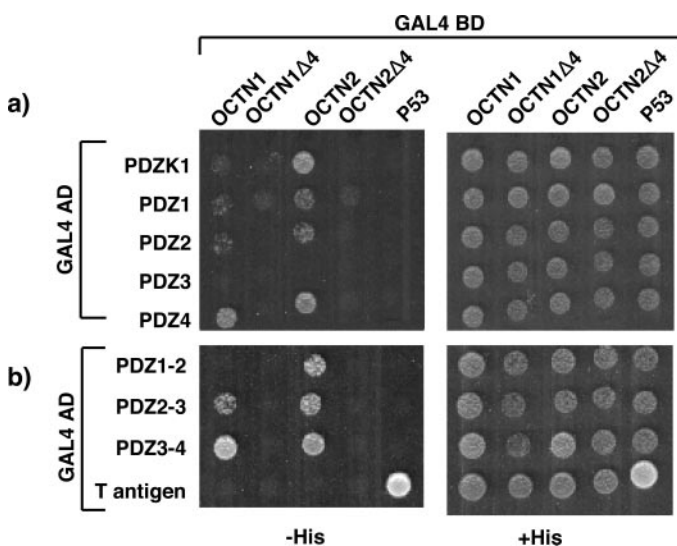
Fig. 4. Yeast two-hybrid analysis for characterization of the interaction of PDZK1 with OCTN1 and OCTN2. Yeast cells were cotransformed with plasmids encoding PDZK1, each PDZ domain (a: PDZ1, PDZ2, PDZ3, and PDZ4) or two sequential PDZ domains (b: PDZ1–2, PDZ2–3, and PDZ3–4) in PDZK1 (subcloned as a fusion protein with GAL4AD into pGADT7 vector) and the C terminus of OCTN1, OCTN2, or their four amino acid-truncated mutants (OCTN1Δ4 and OCTN2Δ4) (subcloned as a fusion protein with GAL4BD in pGBKT7 vector). T antigen and p53 were used in the control experiment. Interaction was indicated by growth on agar plates made with medium without histidine (−His).

Fig. 5. Pull-down of endogenous PDZK1 expressed in mouse kidney brush-border membrane with the recombinant carboxyl terminus of organic cation transporters. Mouse kidney brush-border membrane vesicles were solubilized in radioimmunoprecipitation assay-Y buffer and subjected to pull-down analysis with GST fusion protein bearing the carboxyl terminus of OCTN1, OCTN1 mutant with the carboxyl-terminal four amino acids truncated (OCTN1Δ4), OCTN2, OCTN2 mutant with the carboxyl-terminal four amino acids truncated (OCTN2Δ4), OCT2, and OCT3 or GST alone (GST). The precipitated (interacted) materials were then analyzed by Western blotting using PDZK1 antibody (×30).

fected rat OAT1, which did not interact with PDZK1 in our previous yeast two-hybrid analysis (Kato et al., 2004), and examined the time profile of uptake of *para*-aminohippuric acid, a typical substrate of OAT1; comparable levels of uptake by rOAT1 were observed in each cell lines (data not shown). Thus, PDZK1 specifically promotes the transport activity of OCTN2.

Kinetic analysis of carnitine transport was then performed to examine whether an increase in transport could be caused by an increase in V_{max} and/or a decrease in K_m. Saturable uptake was observed during transient expression of OCTN2 in both HEK293/PDZK1 and HEK293 cells (Fig. 10, a and b). In each cell line, OCTN2-mediated uptake was then examined by subtraction of the uptake observed during transient expression of EYFP vector alone from that observed after YFP-OCTN2 transfection. Eadie-Hofstee plots revealed the

existence of a single component, with a similar slope, for such saturable uptake (Fig. 10c). The obtained values for K_m, V_{max}, and V_{max}/K_m were 3.06 ± 0.31 and 2.70 ± 0.10 μM, 103 ± 8 and 643 ± 17 pmol/mg protein/3 min, and 33.7 and 238 μl/mg protein/3 min in HEK293 and HEK293/PDZK1 cells, respectively.

Discussion

Although much experimental evidence indicates important roles of xenobiotic transporters as barriers against toxic compounds and influx pumps to take up nutrients into the body, little information is available on regulatory proteins which directly interact with the transporters to modulate their function. In the present study, we demonstrated the interaction of PDZK1 with OCTN2, colocalization of the two proteins
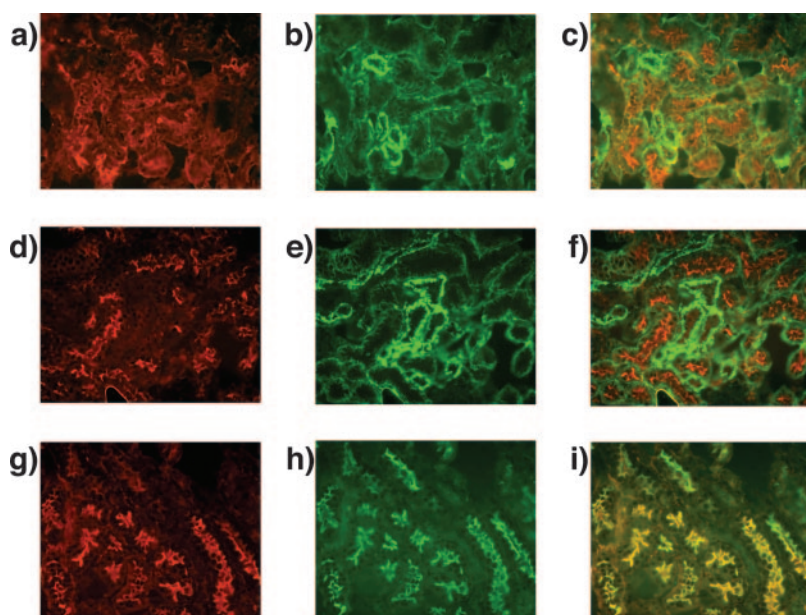
Fig. 6. Immunolocalization of PDZK1 and OCTN2 in mouse kidney. Cryosections (10 μm) of mouse kidney were double-stained with affinity-purified rabbit antiserum against mouse OCTN2 (a and h), mouse monoclonal antibody against Na⁺, K⁺, and ATPase (b and e), and rat antiserum against mouse PDZK1 (d and g). Overlay images (c, f, and i) show that both OCTN2 and PDZK1 are localized at the apical membrane of proximal tubular cells. Magnification, ×400.
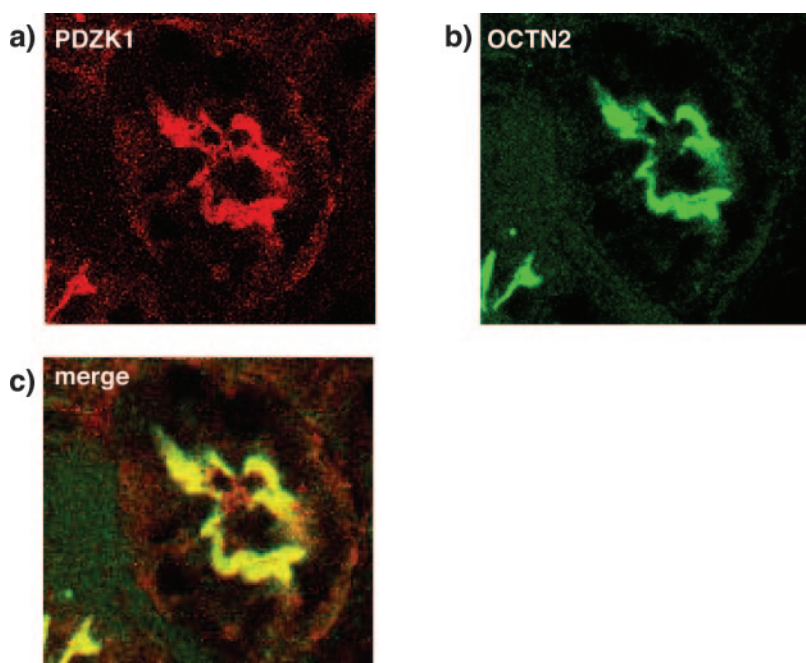
Fig. 7. Confocal microscopy analysis for localization of PDZK1 and OCTN2 in mouse kidney. Cryosections of mouse kidney were double-stained with affinity-purified rabbit antiserum against mouse OCTN2 (a) and rat antiserum against mouse PDZK1 (b). The overlay image (c) shows that OCTN2 and PDZK1 were colocalized at the apical membrane of proximal tubular cells. Magnification, ×1000.

on apical membranes of kidney proximal tubules and re-markable stimulation of the carnitine transport activity of OCTN2 by PDZK1. Yeast two-hybrid analysis suggested that the interaction between PDZK1 and OCTN2 is direct. The essential requirement of the last four amino acids at the C terminus of OCTN2 was confirmed in both pull-down and yeast two-hybrid experiments. The deletion of these four amino acids resulted in loss of the stimulatory effect of PDZK1 on OCTN2 function. These results suggest that PDZK1 is a functional regulator of OCTN2. To our knowledge, this is the first report of a regulatory factor of OCTN2 that directly interacts with OCTN2.

OCTN2 has unique transporting characteristics. It is in-volved in both Na^+-dependent influx of carnitine and Na^+-independent efflux of organic cations (Ohashi et al., 1999; Tamai et al., 2001). Its essential role in such bidirectional transport of nutrients and xenobiotics has been suggested already by the fact that juvenile visceral steatosis mouse with a genetic deficiency in OCTN2 exhibit systemic carni-tine deficiency and reduced renal excretion of exogenously administered tetraethylammonium (Ohashi et al., 2001). Therefore, colocalization of PDZK1 with OCTN2 on the apical surface of renal tubules (Figs. 6 and 7) and pull-down of PDZK1 in renal BBMVs with the OCTN2 C terminus (Fig. 5) suggest that PDZK1 could be involved in the regulation of reabsorption of carnitine via OCTN2 in kidney.

The novel finding in the present study of a functional regulator that induces a dramatic increase in OCTN2-medi-ated carnitine transport activity may have various biological implications when we consider the importance of carnitine for maintaining homeostasis in the body. For example, both PDZK1 and OCTN2 are expressed in testis (Kocher et al., 1998; Tamai et al., 1999, 2000; Wang et al., 2000). OCTN2 is localized in sperm and is proposed to be involved in concen-trative carnitine uptake into testis (Xuan et al., 2003). Be-cause epididymal and spermatozoal carnitine levels affect spermatozoan maturation, motility, and fertility, not only OCTN2 itself but also its adaptor protein PDZK1 may affect the function of sperm. The most well-known cases demon-strating the importance of such an adaptor protein in homeo-static regulation include low-density lipoprotein (LDL) recep-tor-mediated endocytosis, which is the primary pathway for the clearance of circulating LDL and cholesterol. Hypercho-lesterolemia is caused not only by mutation in the LDL receptor but also by mutation in its adaptor protein, ARH, which leads to autosomal recessive hypercholesterolemia (Norman et al., 1999; He et al., 2003). Targeted disruption of the PDZK1 gene in mice also causes hypercholesterolemia, altering lipoprotein metabolism and increasing plasma total and high-density lipoprotein cholesterol, possibly caused by the almost complete loss of expression of liver scavenger receptor class B type I, which interacts with PDZK1 (Kocher et al., 2003). Thus, further analysis regarding the genetic mutation and/or immaturation of PDZK1 may be required to examine its significance in relation to homeostatic regulation by carnitine.

PDZK1 has four PDZ domains, and according to the present results, the C terminus of OCTN2 can bind to PDZ2
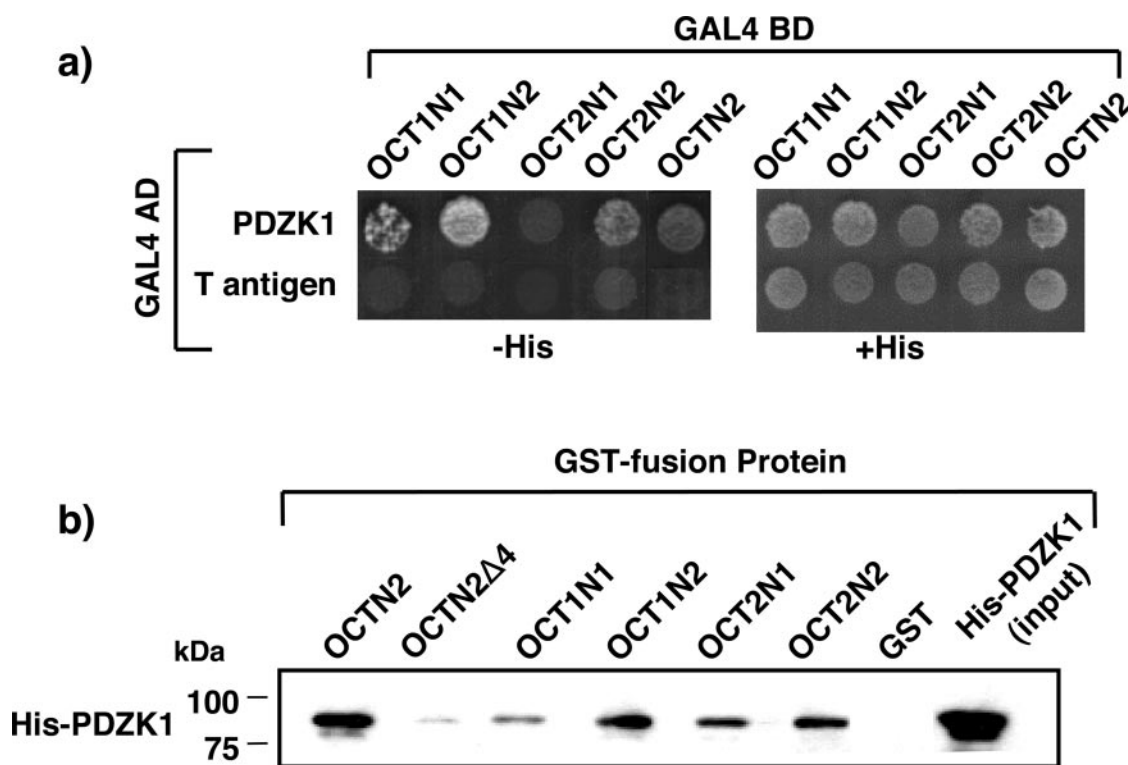

Fig. 8. Interaction of PDZK1 with chimeric constructs of OCTs with C-terminal four amino acids replaced with those of OCTNs. a, yeast cells were cotransformed with plasmids encoding PDZK1 (subcloned as a fusion protein with GAL4AD into pGADT7 vector) and chimeric constructs of OCTs with C-terminal four amino acids replaced with those of OCTNs (OCT1N1, OCT1N2, OCT2N1, and OCT2N2) (subcloned as a fusion protein with GAL4BD in pGBKT7 vector). T antigen was used in the control experiment. Interaction was indicated by growth on agar plates made with medium without histidine (−His). b, recombinant His$_6$-tagged PDZK1 was pulled down with GST fusion protein bearing the carboxyl terminus of OCTN2, OCTN2Δ4, chimeric constructs of OCTs with C-terminal four amino acids replaced with those of OCTNs (OCT1N1, OCT1N2, OCT2N1, and OCT2N2), or GST. The precipitated materials were then analyzed by Western blotting using anti-His$_5$ antibody. Magnification, ×30.

and PDZ4, with the last four amino acids of OCTN2 being essential for this binding (Fig. 4a). The binding of PDZ2 with the C terminus of OCTN1 has already been reported by Gisler et al. (2003), and this seems reasonable, considering that both OCTN1 and OCTN2 have the same C-terminal three amino acids (the last four amino acids of OCTN1 and OCTN2 are -ITAF and –STAF, respectively) which are conserved among mouse, rat, and human orthologs. In addition, because PDZ4 alone and PDZ3–4 can bind to the C terminus of OCTN1 and OCTN2 even more strongly than to other single or sequential PDZ domain constructs, and this binding also requires the C-terminal four amino acids, these transporters may also bind to PDZ4 (Fig. 4b). Although the physiological significance of the involvement of such multiple PDZ domains is still unknown, it should be noted that PDZK1 also regulates the function of cystic fibrosis transmembrane conductance regulator, with multiple PDZ domains in PDZK1 being essential for this (Wang et al., 2000). Therefore, further analysis is necessary to clarify the structural basis of the regulatory mechanism. The similar interaction patterns of PDZK1 with OCTN1 and OCTN2 lead to the question of how the interaction with the two transporters is controlled under physiological conditions because both OCTN1 and OCTN2 are similarly expressed on apical membranes of proximal tubules (Tamai et al., 2004). It should also be noted that as

shown in Fig. 4a, the C terminus of OCTN1 could bind to PDZ1 and PDZ2 but not to PDZ1–2. Such an unexpected behavior may suggest the limitation of the experimental system, which isolated only one or sequential PDZ domain(s). Therefore, further studies may be required to prove the involvement of each PDZ domain in the interaction with the OCTN transporters.

PDZ4 is also known to be involved in the interaction of PDZK1 with MAP17, which is highly expressed on the apical surface of renal tubules and various carcinogenic cells (Kocher et al., 1995, 1999). Although the physiological function of MAP17 has not yet been fully characterized, expression of MAP17 is essential for the apical expression of PDZK1 in opossum kidney cells, and loss of MAP17 results in the internalization of PDZK1 (Pribanic et al., 2003). On the other hand, MAP17 is suggested to be involved in turnover of PDZK1 in a proteosome-independent manner, regulating the expression level of PDZK1 (Silver et al., 2003). Thus, MAP17 and PDZK1 could be involved not only in regulation of transport function, but also in that of expression of OCTN2. In the present study, we observed similar cell-surface expression of OCTN2 in HEK293/PDZK1 and HEK293 cells (Fig. 9b). Such similar expression of OCTN2 was also confirmed in the Western blot analysis of membrane fractions (Fig. 9a). Therefore,
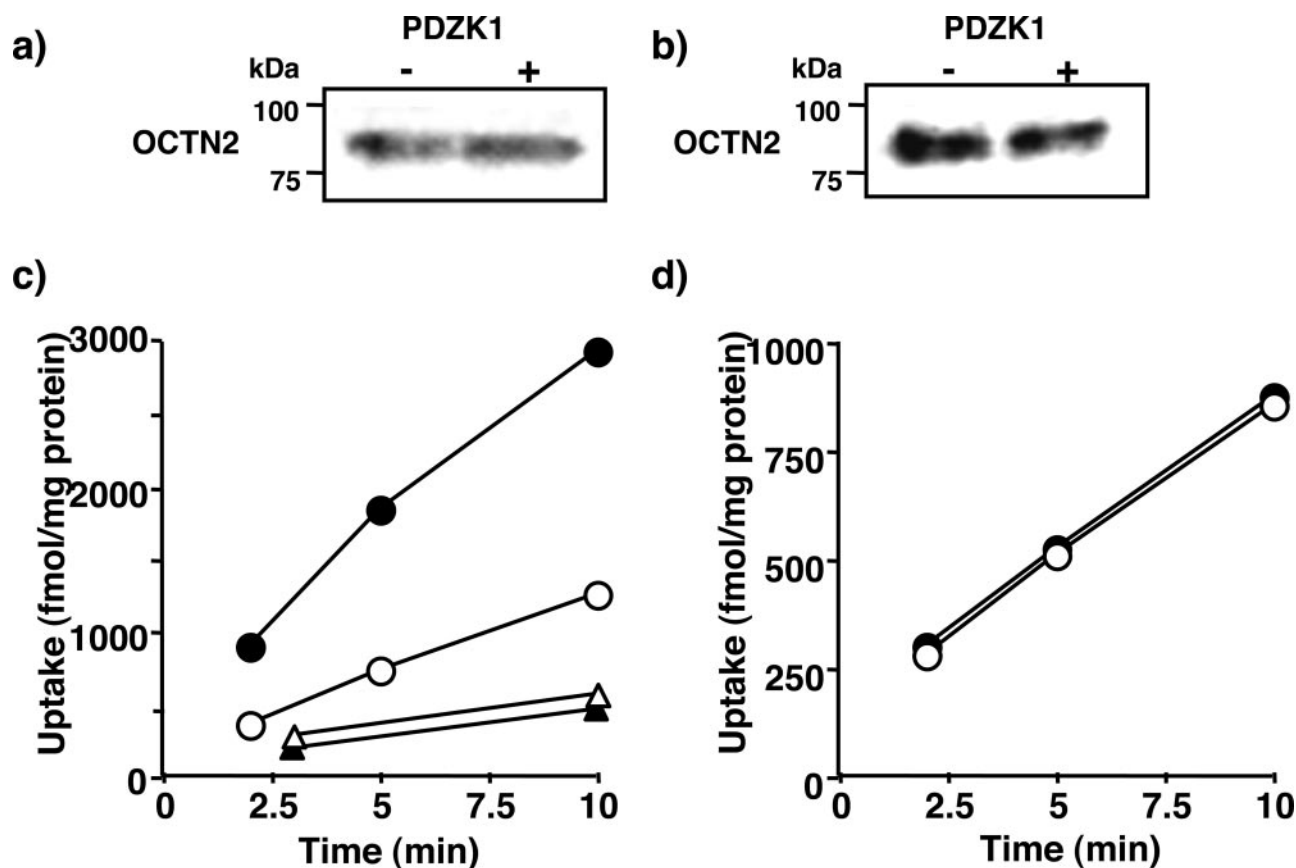

Fig. 9. Effect of PDZK1 on expression of YFP-OCTN2 (a and b) and transport activity of YFP-OCTN2 (c) or its mutant with the carboxyl-terminal four amino acids truncated (YFP-OCTN2Δ4) (d). a, HEK293 or HEK293/PDZK1 cells were transiently transfected with YFP-OCTN2, and Western blot analysis was performed for cellular membrane fraction using anti-green fluorescent protein antibody. b, HEK293 or HEK293/PDZK1 cells were transiently transfected with YFP-OCTN2, and biotinylation of cell-surface membrane was performed, followed by collection of the biotinylated proteins using streptavidin agarose and Western blot analysis as in a. c and d, HEK293 (○) or HEK293/PDZK1 (●) cells were transiently transfected with YFP-OCTN2 (c) or YFP-OCTN2Δ4 (d), and uptake of L-[³H]carnitine (12.5 nM) was measured. As a control experiment, HEK293 (△) or HEK293/PDZK1 (▲) cells were transiently transfected with pEYFP vector alone in c. The results are shown as mean ± S.E.M. of three determinations. Where error bars are not shown, they are smaller than the symbols.

the increase in V_{max} of carnitine transport in HEK293/PDZK1 cells (Fig. 10) seems not to be explained by an increase in OCTN2 expression but can be accounted for by an increase in substrate translocation activity. The reason for the increase in transport activity is still unknown, and further studies are required to examine any post-translational modifications such as the phosphorylation status of OCTN2.

It has long been speculated that the C-terminal PDZ binding motif can account for the localization of transporters either on basolateral or apical membranes in kidney, because all the apical transporters have such a motif in their C terminus (Russel et al., 2002). The C termini of OCT1 (-PSGT) and OCT2 (-IPLN) have no PDZ binding motif, whereas OCT3 (-RSHL), OCTN1, and OCTN2 do have the motif in their C terminus. The interaction with PDZ proteins found in the present study (Fig. 1) is consistent with the presence of such a motif in each transporter. In addition, OCT1 and OCT2 are basolateral transporters, whereas OCTN1 and OCTN2 are expressed in apical membranes of renal tubules (Sweet and Pritchard, 1999; Karbach et al., 2000; Tamai et al., 2001, 2004). Therefore, the present findings imply a possible role of PDZ-transporter interaction in the localization of transporter proteins. Nevertheless, the multiple interactions of both OCTN1 and OCTN2 with various PDZ proteins (Figs. 2 and 3) also indicate that the physiological relevance of such interactions is probably not limited to a single biological aspect. NHERF1 and NHERF2, for example, are involved in the cAMP-dependent down-regulation of NHE3 and affect the intracellular trafficking of NHE3. Thus, there could be many other aspects of the PDZ-OCTN2 interaction than the functional regulation identified in the present study.

In conclusion, the present report has identified a functional regulator of OCTN2 that acts through direct interaction between PDZ domains and the C terminus of OCTN2. Further work is needed to establish the physiological and pharmaco-
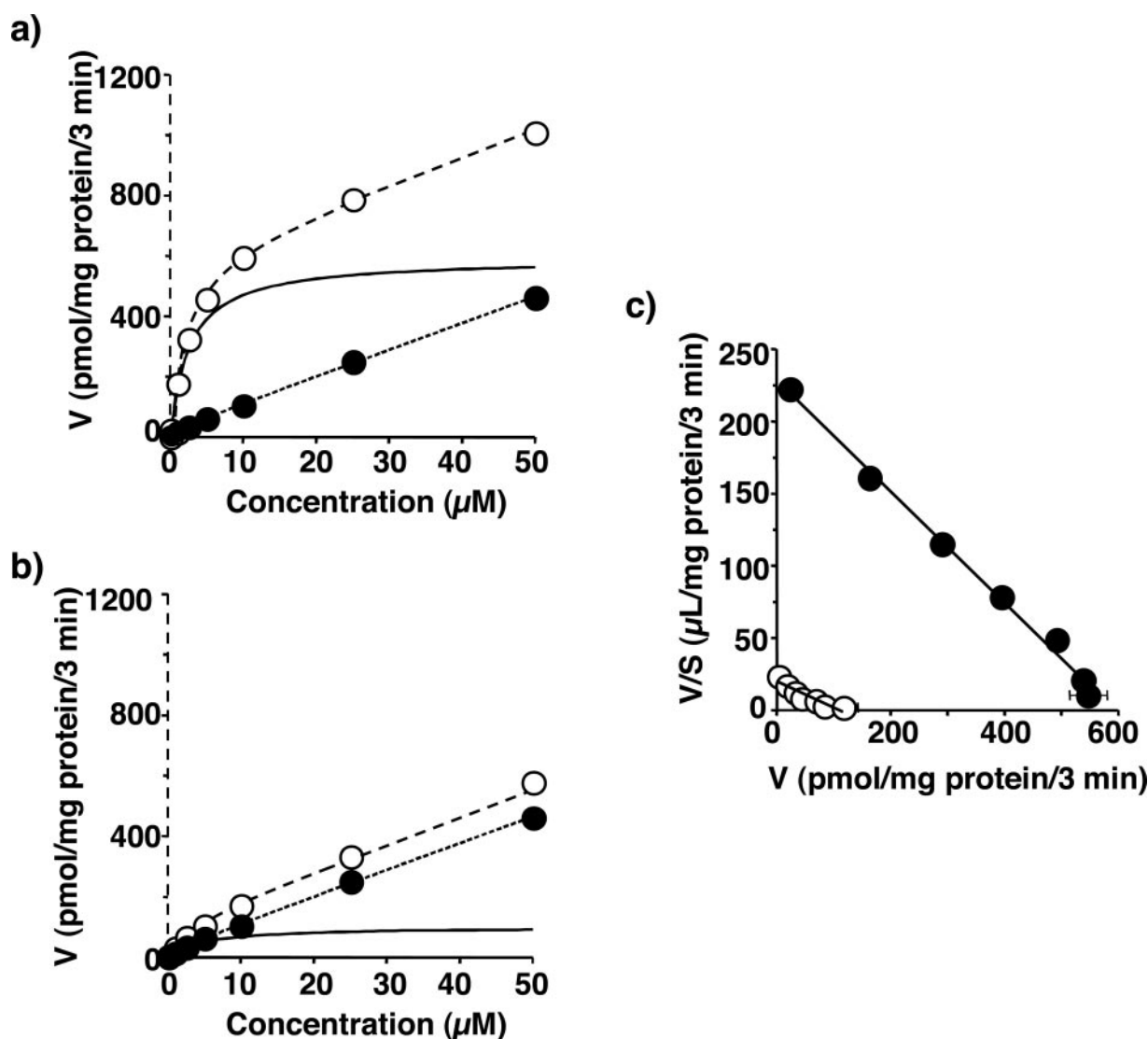

Fig. 10. Effect of PDZK1 on concentration dependence of L-[³H]carnitine uptake by YFP-OCTN2. HEK293/PDZK1 (a) or HEK293 (b) cells were transiently transfected with YFP-OCTN2 (○) or pEYFP vector alone (●), and uptake of L-[³H]carnitine was then measured for 3 min at 37°C. Solid lines represent the OCTN2-mediated uptake after subtraction of the background uptake obtained from pEYFP vector-transfected cells from the total uptake obtained from OCTN2-transfected cells. c, each OCTN2-mediated uptake in HEK293/PDZK1 (●) or HEK293 (○) cells was analyzed by means of the Eadie-Hofstee plot (c). The results are shown as mean ± S.E.M. of three determinations. Where error bars are not shown, they are smaller than the symbols.

logical importance of such adaptor proteins in the disposition of xenobiotics and nutrients in the body.

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