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PDZ adaptor protein PDZK2 stimulates transport activity of organic cation/carnitine transporter OCTN2 by modulating cell-surface expression

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Effect of PDZK2 on organic cation/carnitine transporters (OCT/OCTNs)

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Abbreviations: OCTN, organic cation/carnitine transporter; NHERF, Na+/H+ exchanger regulatory factor; SLC, solute carrier; GST, Glutathione S-transferase

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ABSTRACT

A part of organic cation transporter families (OCT3, OCTN1 and OCTN2) have recently been identified to physically interact with PDZ (PSD95, Dlg and ZO1) domain containing proteins, although the physiological relevance of such interaction has not yet been fully examined. Here we have examined stimulatory effect of PDZK2 (also named NaPi-Cap2 and intestinal and kidney-enriched PDZ protein, IKEPP) on those cation transporters. In HEK293 cells, coexpression with PDZK2 increased the uptake of carnitine by OCTN2 with minimal effect on its substrate recognition specificity, but not for transport activity of OCT3 or OCTN1. The stimulatory effect of PDZK2 on OCTN2 was compatible with approximately two times increase in transport capacity and can be accounted for by the increase in cell-surface expression of OCTN2. Coexpression of PDZK2 did not affect carnitine transport activity of OCTN2 with deletion of the last four amino acids that were found to be important for the interaction, suggesting involvement of physical interaction of the two proteins in increase of cell-surface expression of OCTN2. In mouse kidney, colocalization of PDZK2 and OCTN2 was predominantly located in the region which was close to, but not the same as, the surface of apical membranes where OCTN2 alone was observed, suggesting the existence of OCTN2 in subapical compartment that interacts with PDZK2. The present data have thus proposed “intracellular pool” for OCTN2 that may be relevant to the stabilization of cell-surface expression of OCTN2, thereby increasing transport activity for carnitine.

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The kidney plays an important role in the elimination of numerous hydrophilic xenobiotics, including drugs, and other endogenous compounds. Many of them are secreted into the urine via renal epithelial cells and therefore, it seems reasonable to suppose that they are actively transported across plasma membranes via specialized carrier-mediated transport mechanisms. In fact, a number of transporting proteins were identified and shown their expression on basolateral and apical membranes of renal epithelial cells. Among them, organic cation transporter OCT/OCTN family has been suggested to be involved in the secretion of organic cations. Two OCT family member, OCT1 (SLC22A1) and OCT2 (SLC22A2) are localized on basolateral membranes whereas two OCTN family member, OCTN1 (SLC22A4) and OCTN2 (SLC22A5) are expressed predominantly at the apical membrane of cortical proximal tubular epithelial cells in kidneys of rodents (Jonker and Schinkel 2004; Koepsell and Endou 2004; Lee and Kim 2004; Sai and Tsuji 2004; Wright 2005). Both OCT1 and OCT2 have been shown to mediate the electrogenic transport of a broad range of structurally diverse cationic compounds neurotransmitter (Jonker and Schinkel 2004; Koepsell et al., 2003; Wright 2005). These include the prototypic organic cation tetraethylammonium (TEA), antidiabetics, neurotoxins, and a variety of endogenous compounds such as choline, monoamine and neurotransmitter (Jonker and Schinkel 2004; Koepsell et al., 2003; Lee and Kim 2004; Sai and Tsuji 2004; Wright 2005). OCTN1 is thought to be a multispecific and pH-dependent organic cation transporter, which presumably functions as a proton/organic cation antiporter and/or organic cation/organic cation antiporter (Tamai et al., 1997; Yabuuchi et al., 1999). OCTN1 also transports ergothioneine and stachydrine, both being zwitter ionic compounds (Grundemann et al., 2005). OCTN2 accepts various organic cations including TEA, verapamil, pyrilamine and quinidine as substrates (Ohashi et al., 2001, 2002). OCTN2 is physiologically important to maintain plasma concentration of carnitine, which plays an important role in the transport of long-chain fatty acids across the

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mitochondrial inner membrane for β-oxidation and energy metabolism (Wu et al., 1998; Tamai et al., 1998; Ohashi et ai., 1999; Wang et al., 1999; Nezu et al., 1999). Such broad substrate specificity of OCTN1 and OCTN2 suggests a crucial role of this transporter in facilitating the elimination of xenobiotics.

Despite their pharmacological importance, research on regulatory mechanisms that directly interact with these OCT/OCTN family members has just recently been started. One of the key issues regarding their regulatory proteins may be direct interaction of OCT/OCTN family members expressed on apical membranes of renal epithelial cells (OCTN1, OCTN2 and OCT3) with PDZ (PSD-95/Dlg/ZO-1) domain containing proteins. PDZ domains are modular protein interaction domains that bind in a sequence-specific fashion to short C-terminal peptides or internal peptides that fold in a β-finger (Karthikeyan et al., 2001; Hillier et al., 1999; Harris et al., 2001). We have recently reported a number of specific interactions between apically expressed OCT/OCTN family members and PDZ proteins including PDZK1, PDZK2 (also known as NaPiCap2 and intestinal and kidney-enriched PDZ protein, IKEPP), Na^+/H^+ exchanger regulatory factor (NHERF) 1 and NHERF2 (Kato et al., 2004, 2005). Both PDZK1 and PDZK2 have four PDZ domains in their structure, whereas NHERF1 and NHERF2 have two. These PDZ proteins had been originally identified to directly interact with inorganic ion transporters such as Na^+/H^+ exchanger 3, Na^+-dependent phosphate transporter (NPT) 2a, multidrug resistance-associated protein (MRP) 2 and cystic fibrosis transmembrane conductance regulator (CFTR) (Weinman et al., 1993; Custer et al., 1997; Yun et al., 1997; Kocher et al., 1999; Russel et al., 2002; Gisler et al., 2001; Wang et al., 2000).

Several reports have suggested that these PDZ proteins play an essential role in intracellular signaling not only as protein-protein recognition scaffolding modules (Biber *et al*.,

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2004; Brone and Eggermont 2005), but also as a functional regulator. For example, the activity of CFTR might be regulated by formation of CFTR dimmer through the interaction with PDZK1 (Wang et al., 2000). The association of PDZK1 with urate-anion exchanger 1 (URAT1/SLC22A12) enhanced urate transport activity possibly via the increase in the stability of URAT1 on cell-surface (Anzai et al., 2004). Our previous analysis also indicated PDZK1 as a functional regulator of OCTN2 via a direct interaction with the C-terminus (Kato et al., 2005). The increase in OCTN2-mediated carnitine transport activity in the presence of PDZK1 is accounted for by approximately 6-times increase in transport capacity, but not by the change in cell-surface expression level of OCTN2 (Kato et al., 2005). The broad substrate specificity of OCTN2 and mouse homolog of URAT1 (Imaoka et al., 2004) for organic cations and anions, respectively, may imply significant role of the PDZ protein in disposition of many types of therapeutic agents which are substrates of the transporters interacting with PDZK1. Actually, PDZK1 has also recently been demonstrated to be required for basolateral expression of hepatic organic anion transporter OATP1A1, and *pdzk1* gene knockout hinders systemic elimination of an organic anion (Wang et al., 2005). Thus, these PDZ proteins could play a role as a regulatory mechanism for at least a certain types of membrane proteins. However, regarding OCT/OCTN family members, information on the functional regulation has been obtained only for PDZK1, but pharmacological roles of other PDZ proteins that directly interact with OCT/OCTN family is still unknown.

Among those PDZ proteins, PDZK2 is homologous to PDZK1 and is expressed in kidney and intestines (Scott et al., 2002). PDZK2 was first identified as a protein that interacts with the C-terminus of NPT2a, and is predominantly localized in the subapical compartment in mouse renal proximal tubules (Gisler et al., 2001). PDZK2 is also localized on apical membranes of small intestinal epithelial cells in human (Scott et al., 2002). Our previous yeast two-hybrid screening has revealed that not only PDZK1, but also PDZK2 can interact with C-terminus of

OCTN1 and OCTN2 (Kato et al., 2004). In addition, PDZK2, but not PDZK1 was specified to interact with C-terminus of OCT3 (Kato et al., 2004; Kato et al., 2005). Despite of such available information on physical interaction with OCT/OCTN family, little is known about possible functional modulation of the transporters with PDZK2. In addition, details in characteristics of the interaction between PDZK2 and OCT/OCTN family i.e., domains and regions within each protein essential for the interaction, has not yet been examined. Therefore, in the present study, we attempted to clarify the modulation of transporting properties and cell-surface expression of OCT/OCTN family exerted by PDZK2. In addition, to understand physiological relevance of such protein-protein interaction, colocalization of PDZK2 and OCTN2 was examined in mouse kidney.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibody for OCTN2 was raised as described previously (Tamai et al., 2000). Rat polyclonal antibody for PDZK2 was raised against a recombinant protein of His_6-tagged mouse PDZK2. Monoclonal antibody against Na^+/K^+ ATPase, His_5 and GFP were acquired from Upstate Biotechnology (Lake Placid, NY), Clontech (Palo Alto, CA) and Roche Diagnostics (Basel, Switzerland), respectively. L-[^{3}H]Carnitine (3.1 Tbq/mmol) and [^{14}C]tetraethylammonium bromide (TEA, 2.0 Gbq/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK) and American Radiolabeled Chemicals, INC (Saint Louis, MO), respectively, whereas [^{3}H]1-Methyl-4-phenylpyridinium (MPP, 3.2 TBq/mmol) was from PerkinElmer (Wellesley, MA). The myc epitope tagged human PDZK2 was subcloned in pcDNA3 (Invitrogen, San Diego, CA) as described previously (Kato et al., 2005). cDNA fragments encoding single or multiple PDZ domains (listed below) in PDZK2 were obtained by PCR amplification: aa 1-132 for PDZ1, aa 146-241 for PDZ2, aa 251-357 for PDZ3, aa 398-505 for PDZ4, aa1-241 for PDZ1-2, aa146-357 for PDZ2-3 and aa251-505 for PDZ3-4. All these cDNAs were subcloned into pGADT7 (Clontech) and pET30 (Novagen) plasmids between the EcoRI and XhoI sites. cDNA fragments encoding the C-terminal 41, 41, 39, 44, and 48 amino acids of human OCT1, OCT2, OCT3, OCTN1 and OCTN2, respectively, were subcloned into both pGBKT7 (Clontech) and pGEX6P-1 (Amersham Biosciences, Buckinghamshire, UK) plasmids between the EcoRI and SalI sites. The full sequences of all the inserts were verified. Glutathione S-transferase (GST) fusion proteins and His_6-tagged PDZK1 were obtained from E. Coli (BL-21 strain) transformed with pGEX6P-1 and pET30 constructs, respectively, according to the manufacturer's instructions. cDNA encoding full-length human OCTN2 was subcloned into

pEYFP-C1 vector (Clontech) whereas cDNA encoding human OCTN1 and rat OCT3 were subcloned into pcDNA3 vector (Invitrogen).

Transport studies in HEK293 cells stably expressing PDZK2

HEK293 cells were transfected with myc-tagged full-length human PDZK2, and stably transfected cells were selected by adding G418 (Sigma) to the culture medium to obtain HEK293/PDZK2 cells. The cDNA encoding human OCTN1, YFP fusion protein with human OCTN2 or rat OCT3 was then transiently transfected according to the calcium phosphate precipitation method (Tamai et al., 2000). At 48 hr after transfection, cells were harvested and suspended in a transport medium (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, pH 7.4). Uptake experiment was then performed according to the procedures of the silicone-oil layer method as described previously (Tamai et al., 2000). In the present study, to routinely confirm transfection efficiency at each cell line in each experiment, we used the YFP-OCTN2 fusion construct. The observed intensity of fluorescence derived from YFP in HEK293 and HEK293/PDZK2 cells was almost the same level.

Western blot analysis for YFP-OCTN2 expressed on cell-surface

At 48 hr after transfection of YFP-OCTN2, the cells were incubated with sulfo-NHS-LC-Biotin (Pierce, Rockford, IL), and the biotinylated fraction was captured by immobilized streptavidin, followed by Western blot analysis using anti-OCTN2 antibody as described previously (Kato et al., 2005).

Immunocytochemical analysis

Frozen sections of mouse kidney were prepared as described previously (Kato et al., 2005). The sections were first heated to 92 ^{o}C in the RETRIEVE-ALL (SIGENT Pathology Systems Inc., Dedham, MA) buffer. They were then incubated with a mixture of antibodies for overnight at 4 ^{o}C, and subsequently incubated with secondary antibodies (Alexa Fluor 594 goat anti-rat and anti-mouse IgG conjugates or Alexa Fluor 488 anti-rabbit and anti-rat IgG conjugates, Molecular Probes Inc., 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 (Carl Zeiss, Jena, Germany).

Yeast two-hybrid analysis

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

Pull-down assays

GST C-terminus fusion proteins (50 μg) were incubated with purified His_6-PDZK2 and glutathione-Sepharose 4B at 4 ^{o}C for 3 hr in PBS (1 ml) supplemented with 0.5 mM dithiothreitol, protease inhibitors (Complete EDTA-free, Roche Diagnostics) and 1% (delete 0.05%) Triton X-100. The suspension was then washed twice with ice-cold PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with antibody to the His_5 tag (Amersham Biosciences) using polyvinylidene difluoride membrane (Immobilon, Millipore,

Bedford, MA). It was then reacted with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences). The protein was detected with the ECL-plus Western-blotting detection system (Amersham Biosciences).

Immunoprecipitation assay

Immunoprecipitation using anti-c-myc antibody was performed as described previously (Kato et al., 2004). Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-GFP antibodies.

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RESULTS

Effect of PDZK2 on transport activity of OCTN1, OCTN2 and OCT3

To examine whether function of OCTN1, OCTN2 and OCT3 is modulated by PDZK2, uptake study was first performed in HEK293/PDZK2 cells transiently expressing each transporter in the present study. In both HEK293/PDZK2 and HEK293 cells, uptake of [^{14}C]TEA was higher after transient transfection of OCTN1 than that after transfection of vector alone (Fig. 1A). Uptake of [^{14}C]TEA after the OCTN1 transfection in HEK293/PDZK2 cells was almost comparable with that in HEK293 (Fig. 1A). Similar uptake was also observed between both cell lines after transfection of OCTN1 with the last four amino acids deleted (OCTN1Δ4) (Fig. 1B). On the other hand, the uptake of L-[^{3}H]carnitine by OCTN2 in HEK293/PDZK2 cells was higher than that in HEK293 cells (Fig. 1C), whereas the uptake by OCTN2Δ4 was almost identical between each cell line (Fig. 1D). Uptake of [^{3}H]MPP by OCT3 was comparable between HEK293/PDZK2 and HEK293 cells (Fig. 1E).

Effect of PDZK2 on cell-surface expression and kinetic parameters for carnitine transport of OCTN2

Kinetic analysis was then performed for carnitine transport by OCTN2 both in HEK293/PDZK2 and HEK293 cells to clarify whether the effect of PDZK2 is on the transport affinity or capacity (Fig. 2A). Saturable uptake was observed after a transient transfection of OCTN2 in both HEK293/PDZK2 and HEK293 cells (Fig. 2A). In each cell line, Eadie-Hofstee plots revealed the existence of a single component, with a similar slope, for such saturable uptake (Fig. 2A). The obtained values were K_m 2.39 ± 0.24 and 2.41 ± 0.35 μM, V_{max} 40.6 ± 2.3 and 88.2 ± 7.5 pmol/mg protein/3 min, and V_{max}/K_m 17.0 and 36.6 mL/mg protein/3 min in HEK293 and

HEK293/PDZK2 cells, respectively. To understand the possible reason for the increase in the V_{max} value in the presence of PDZK2, cell-surface expression level of OCTN2 was examined in a biotinylation study (Fig. 2B). The band intensity at 84 kD, which corresponded to YFP-OCTN2, was higher in HEK293/PDZK2 than HEK293 cells in the Western blot analysis of biotinylated proteins, whereas endogenous transferrin receptor, which was also measured as a control experiment, exhibited almost comparable expression (Fig. 2B).

Effect of PDZK2 on substrate recognition specificity of OCTN2

We next examined effect of various compounds on OCTN2-mediated L-[^{3}H]carnitine uptake in HEK293/PDZK2 and HEK293 cells (Fig. 3) with an aim to examine an effect of PDZK2 on substrate recognition specificity of OCTN2. Although our previous analysis revealed functional stimulation of OCTN2 by PDZK1, the change in substrate recognition specificity of OCTN2 by PDZK1 has not yet been clarified (Kato et al., 2005), Therefore, similar experiments were also performed in HEK293/PDZK1 cells in the present study. Quinidine, valproic acid and pyrilamine inhibited OCTN2-mediated uptake of L-[^{3}H]carnitine in a concentration-dependent manner (Fig. 3). Such inhibition curve was almost similar between each cell line (Fig. 3). Unlabeled L-carnitine, TEA, verapamil, procainamide and N-methylnicotinamide also inhibited L-[^{3}H]carnitine uptake, and such inhibitory effect was almost similar between each cell line (Table 1).

Partial colocalization of OCTN2 with PDZK2 in mouse kidney

To support the physiological significance of the interaction between PDZK2 and OCTN2, both were stained in immunohistochemical analysis, and their colocalization in mouse kidney was examined (Fig. 4). Immunostaining with antibody that was raised against full-length of

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mouse PDZK2 revealed reactive proteins in proximal and distal tubules where distinct staining was observed with antibody against Na^+/K^+-ATPase (Fig. 4A, 4B, 4C). Moreover PDZK2 was localized at apical membrane in proximal and distal tubules, and even in cytoplasmic region of the proximal tubules. Both immunoreactive proteins for PDZK2 (Fig. 4D, 4G) and OCTN2 (Fig. 4E, 4H) antibodies were detected in apical membranes, and were only partially colocalized (Fig 4F, 4I). Such colocalization was observed in the region that was close to, but not the same as, the surface of apical membranes where OCTN2 alone was observed (Fig. 4F, 4I).

Characterization of the interaction of PDZK2 with C-terminus of OCT/OCTN family

Our previous analysis both in yeast two-hybrid and pull-down analyses revealed specific interaction of PDZK1 with OCTN1 and OCTN2, and PDZK2 with OCTN1, OCTN2 and OCT3 (Kato et al., 2004; 2005), although details of the characteristics of the interaction have not yet been examined for PDZK2. We first purified His_6-tagged PDZK2 and performed pull-down studies with GST fusion of OCT/OCTN family (Fig. 5A) in the present study. PDZK2 was pull-downed with GST fusion of OCTN1, OCTN2 and OCT3 (Fig. 5A). This result was compatible with the previous finding (Kato et al., 2005). Neither OCT1, OCT2 or GST alone could interact with PDZK2 (Fig. 5A). Deletion of the last four amino acids in OCTN1 and OCTN2 decreased the interaction potential with PDZK2, although the weak interaction was still observed for OCTN1Δ4 and OCTN2Δ4 (Fig. 5A).

The interaction between PDZK2 and C-terminus of OCTN1, OCTN2 and OCT3 was confirmed in yeast two-hybrid analysis in which the deletion of the last four amino acids again reduced the interaction with PDZK2 (Fig. 5B). Both OCTN1 and OCTN2 interacted with a single PDZ domain PDZ1 and PDZ3 (Fig. 5B), the interaction being decreased for OCTN1 and OCTN2

with the last four amino acids of the C-terminus deleted (Fig. 5B). All of the two sequential PDZ domains (PDZ1-2, PDZ2-3, PDZ3-4) examined interacted with OCTN1 and OCTN2 (Fig. 5B). In contrast, only PDZ1 and PDZ1-2 could bind to the C-terminus of OCT3 (Fig. 5B). These results suggest that both PDZ1 and PDZ3 in PDZK2 are responsible for the interaction with OCTN1 and OCTN2, whereas PDZ1 is involved in the interaction with OCT3. We also performed immunoprecipitation assay in HEK293/PDZK2 cells transiently transfected with YFP-OCTN2 to confirm the interaction between PDZK2 and OCTN2 as full-length proteins. A band immunoreactive with anti-GFP antibody was observed after immunoprecipitation with anti-c-myc antibody in HEK293/PDZK1 and HEK293/PDZK2 cells, but not in HEK293 cells, demonstrating physical interaction of OCTN2 with PDZK1 and PDZK2 (Fig. 5C).

DISCUSSION

Organic cation transporter OCT/OCTN family has been suggested to be involved in renal secretion of various types of organic cations (Jonker and Schinkel 2004; Koepsell and Endou 2004; Lee and Kim 2004; Sai and Tsuji 2004; Wright 2005). Therefore, regulatory mechanism(s) for those transporters could be important to understand renal disposition of therapeutic agents. It is noteworthy that PDZ domain containing proteins including PDZK2 has an interaction potential with various types of membrane transporters (Hegedus et al., 2003; Kato et al., 2004). Therefore, PDZK2 could be involved in regulatory mechanisms for various types of transporters and, therefore, related to renal disposition of many types of substrate compounds of those proteins. PDZK2 was found to increase the transport activity of OCTN2, since the uptake of L-[^{3}H]carnitine by OCTN2 in HEK293/PDZK2 cells was higher than that in HEK293 cells (Fig. 1C). Such stimulatory effect of PDZK2 on transport activity can be presumed to be specific for OCTN2 since PDZK2 did not clearly affect the uptake of L-[^{3}H]carnitine by OCTN2Δ4 (Fig. 1D). Direct interaction potential between PDZK2 and OCTN2 was confirmed both in pull-down studies using purified recombinant proteins and yeast two-hybrid system (Fig. 5), in which the last four amino acids in C-terminus of OCTN2 was important for the interaction. Thus, the present study has suggested that PDZK2 can affect the transport activity of OCTN2. In the present study, to confirm the transfection efficiency at each experiment, we used YFP-OCTN2 fusion construct, instead of native OCTN2. Transport studies were always performed after we confirmed similar intensity of fluorescence of YFP-OCTN2 between each HEK293 and HEK293/PDZK2 cells. Although we cannot neglect possible influence of YFP on expression and/or function of OCTN2, this YFP-OCTN2 construct exhibited similar transport activity of carnitine (Figs. 2, 3) to that exerted by a native OCTN2 (Tamai et al., 1998; Ohashi et al., 1999). Furthermore, the

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immunoprecipitation analysis revealed interaction potential of YFP-OCTN with PDZK2 (Fig. 5C).

The increase in OCTN2-mediated uptake of L-[^{3}H]carnitine by PDZK2 can be accounted for by the two times increase in V_{max} according to the present kinetic analysis (Fig. 2A). Since the K_m for the L-[^{3}H]carnitine transport was almost identical between HEK293/PDZK2 and HEK293 cells (Fig. 2A), PDZK2 does not affect the affinity of L-[^{3}H]carnitine transport, but increases its transport capacity. Western blot analysis for biotinylated fraction in both cell lines revealed higher OCTN2 expression on the surface of HEK293/PDZK2 cells than that of HEK293 cells (Fig. 2B). Thus, PDZK2 increases cell-surface expression of OCTN2, leading to an increase in the V_{max} of L-[^{3}H]carnitine transport. Such molecular mechanism for PDZK2 is in contrast to that for PDZK1 since PDZK1 does not affect cell-surface OCTN2 expression, but increases the V_{max} of L-[^{3}H]carnitine transport without an effect on K_m (Kato et al., 2005). Therefore, transport function of OCTN2 is regulated by multiple mechanisms, i.e., cell-surface stabilization by PDZK2 and direct functional modulation by PDZK1. Considering that OCTN2 plays a predominant role in renal reabsorption of carnitine and secretion of TEA, and that its genetic variation causes systemic carnitine deficiency (Nezu et al., 1999; Ohashi et al., 1999, 2001), it might be reasonable that cell-surface expression and function of OCTN2 are tightly regulated by such multiple PDZ proteins.

Although we have demonstrated that PDZK2 minimally affected transport activity of OCTN1 and OCT3 in HEK293 cells (Fig. 1), the present studies for elucidation of the functional regulation by interactions between PDZK2 and OCTN1 or OCT3 might include some insufficient points. First, there might be slight effect of PDZK2 that cannot be detected by silicone-oil layer method used in the present studies. Second, other factors essential for stimulatory effects of PDZK2 are insufficient and/or not expressed in HEK293 cells, but are expressed in vivo. Third, endogenous proteins expressed in HEK293 cells are enough to appropriately regulate the activity

of OCTN1 and OCT3. Because of these possibilities, the in vitro cell line systems have limitation for assessing functional modulation of transporters by adaptor proteins, and further studies are required to understand effect of PDZK2 on these transporters.

Gisler et al. (2001) has revealed that PDZK1 is strictly associated with the microvilli in the apical membranes of the proximal tubules in mouse kidney, whereas PDZK2 is predominantly located in the subapical compartment but was not detected in the microvilli, the faint immunostaining for PDZK2 being also observed throughout the cytoplasm. Tamai et al. (2004) have demonstrated that both OCTN1 and OCTN2 are expressed on apical membranes of proximal tubules in mouse kidney. These previous reports could be compatible with the finding that PDZK1 is colocalized with OCTN2 on apical membranes (Kato et al., 2005), whereas PDZK2 is only partially colocalized with PDZK2 (Fig. 4). However, in the present confocal microscopy analysis, it is noteworthy that such colocalization was predominantly observed in the region that was close to, but not the same as, the surface of apical membranes where OCTN2 alone was observed. This suggests that OCTN2 has at least two distinct compartments; one is localized on apical surface of the plasma membranes, whereas the other is in close proximity to the cell-surface, most likely in subapical region, and colocalized with PDZK2. The present analysis of colocalization with PDZK2 has thus proposed “intracellular pool” for OCTN2, which could be relevant to the regulatory mechanism exerted by PDZK2. Although it is unlikely that such intracellular transporters are directly involved in drug disposition, the intracellular pool for the transporter may be involved in homeostatic regulation as a reservoir of transporter systems.

Previous findings have suggested important roles of OCTN1 and OCTN2 in renal reabsorption of carnitine and secretion of organic cations (Tamai et al., 1997; 1998; Nezu et al., 1999; Ohashi et al., 2001). Therefore, interaction of therapeutic agents with these transporters may affect such renal handling of endogenous and/or exogenous compounds. For example, inhibition

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of OCTN2-mediated L-[^{3}H]carnitine transport by several drugs including an antiepileptic valproate may be related to drug-induced carnitine deficiency (Ohashi et al., 1999). In this sense it would be important to construct experimental systems by which effect of test compounds on OCTN family can precisely be estimated. From practical point of view, cotransfection of OCTN2 with PDZK1 or PDZK2 may have several advantages as compared with transfection of OCTN2 alone since coexpression of PDZK1 and PDZK2 leads to higher transport activity and/or cell-surface expression of OCTN2 (Figs. 1, 2, Kato et al., 2005). It can be speculated that such cotransfection system may exhibit more physiological function of OCTN family if we consider that both PDZ proteins are colocalized in kidney with OCTN2 (Fig. 4, Kato et al., 2005). On the other hand, since inhibitory effect of various cationic and zwitter ionic drugs on OCTN2 was almost similar among HEK293/PDZK2, HEK293 and HEK293/PDZK1 cells (Fig. 3, Table 1), effect of PDZK2 and PDZK1 on recognition specificity of OCTN2 seems to be minimal in the present study, although further studies should be performed to examine the details in substrate recognition specificity of OCTN2 in the presence or absence of PDZ adaptors and its relevance to that in physiological condition.

It should also be noted that the interaction potential with PDZK2 was much lower for OCTN1Δ4 and OCTN2Δ4, compared with OCTN1 and OCTN2, respectively (Fig. 5). This result suggests that the last four amino acids both in OCTN1 and OCTN2 are important for the interaction with PDZK2. However, the C-terminus of OCTN1Δ4 and OCTN2Δ4 exhibited weak interaction with PDZK2, whereas no obvious interaction was found with PDZK1 (Fig. 5, Kato et al., 2005). In addition, the C-terminus of OCTN2 can interact much more potently with PDZK1 than PDZK2 (Fig. 5, Kato et al., 2005). The first and third PDZ domains in PDZK2 are involved in the interaction with OCTN1 and OCTN2 (Fig. 5) whereas the second and fourth PDZ domains in

PDZK1 are important for the interaction (Kato et al., 2005). Thus, structural requirement for the specific interaction with OCTN family seems to be different between PDZK2 and PDZK1. This may be compatible with the low, at the most 28%, homology between the two clones although both are categorized as simple adaptors due to the presence of just four PDZ domains, without any other significant structural domain, in their structure (Bezprozvanny and Maximov, 2001). The difference between PDZK2 and PDZK1 was also observed in their tissue expression and localization. Although both human and mouse the mRNA expression of PDZK2 was almost exclusively observed in kidney and, at a lesser level, in intestines whereas that of PDZK1 was also found in liver and testis of mouse (Gisler et al., 2001; Scott et al., 2002). The mRNA copy number of PDZK2 was found to be much lower than PDZK1 and OCTN2 in mouse kidney (see Results). All these findings are compatible with a hypothesis that physiological significance of PDZK2 in the function regulation of OCTN2 is, at least partially, different from that of PDZK1. The exact physiological roles of these PDZ proteins should be further examined in experimental terms closer to the physiological condition since all the present findings regarding function of PDZK2 have been obtained *in vitro*. In present study, we performed these *in vitro* experiments. In order to understand the pharmacological roles of PDZK1 and PDZK2, further *in vivo* works will be needed.

In conclusion, the present finding revealed that PDZK2 regulates the function of OCTN2 through a direct interaction at its C-terminus, and the regulatory mechanism includes increase in cell-surface expression level of OCTN2. OCTN2 expressed in renal proximal tubules has at least two distinct compartments, and colocalization with PDZK2 occurs in subapical region. Thus, expression of OCTN2 and its carnitine transporting activity might be regulated by multiple mechanisms exerted by PDZK1 and PDZK2.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1 Effect of PDZK2 on transport activity of OCTN1 (A, B), OCTN2 (C, D) and OCT3 (E)

HEK293/PDZK2 (●) and HEK293 (▲) cells were transiently transfected with OCTN1 (A), OCTN1Δ4 (B), YFP-OCTN2 (C), YFP-OCTN2Δ4 (D) or OCT3 (E), and uptake of [^{14}C]TEA (1 μM) (A, B), L-[^{3}H]carnitine (0.1 μM) (C, D), [^{3}H]MPP (2.5 nM) (E) was examined. Control experiment was also performed in HEK293/PDZK2 (○) and HEK293 (△) cells transfected with vector alone (A, C, and E).

Fig. 2 Effect of PDZK2 on concentration dependence of L-[^{3}H]carnitine uptake by OCTN2 (A) and cell-surface expression of OCTN2 (B)

(A) HEK293/PDZK2 (●) or HEK293 (○) cells were transiently transfected with YFP-OCTN2 or pEYFP-C1 vector alone, and uptake of L-[^{3}H]carnitine (0.1 μM) was then measured for 3 min at 37 ^{o}C. OCTN2-mediated uptake was then calculated by subtraction of the background uptake obtained from pEYFP-C1 vector-transfected cells from the total uptake obtained from OCTN2-transfected cells. The results are shown as Eadie-Hofstee plot and the data represent mean ± S.E.M. of three determinations. When error bars are not shown, they are smaller than the symbols. (B) HEK293 or HEK293/PDZK2 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 using anti-OCTN2 and anti-transferrin receptor antibodies. Data represent typical results in three independent experiments.

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Fig. 3 Inhibitory effect of various compounds on OCTN2-mediated carnitine uptake in HEK293/PDZK2, HEK293 and HEK293/PDZK1 cells

HEK293/PDZK2 (●), HEK293 (○) and HEK293/PDZK1 (■) cells were transiently transfected with YFP-OCTN2, and uptake of L-[^{3}H]carnitine for 3 min was measured in the presence of each compound (mean ± S.E.M. of three determinations). OCTN2-mediated uptake was then calculated by subtraction of the background uptake obtained from pEYFP-C1 vector-transfected cells from the total uptake obtained from OCTN2-transfected cells, and normalized by the uptake in the absence of the inhibitor (241 ± 10, 43.4 ± 1.9 and 410 ± 6 μl/mg protein in HEK293/PDZK2, HEK293 and HEK293/PDZK1 cells, respectively).

Fig. 4 Confocal microscopy analysis for localization of PDZK2 and OCTN2 in mouse kidney.

Cryosections (10 μm) of mouse kidney were double-stained with rat antiserum against mouse PDZK2 (A, D, G), monoclonal antibody against Na^+/K^+-ATPase (B) and affinity-purified rabbit antiserum against mouse OCTN2 (E, H). Overlay image (F, I) shows that both PDZK2 and OCTN2 are partially colocalized, whereas OCTN2 alone was localized at the apical membranes of proximal tubular cells. Magnification, ×400.

Fig. 5 Interaction of PDZK2 with C-terminus of OCT/OCTN family

In panel A, His_6-tagged PDZK2 or PDZK1 (50 μg of each) was incubated with 50 μg of GST fusion protein with C-terminus of each OCT/OCTN family protein or their four amino acid-truncated mutants (OCTN1 Δ4 and OCTN2 Δ4). The interacted His_6-tagged PDZK1 was captured by glutathione Sepharose, and precipitated materials were subjected to SDS-PAGE and

subsequent Western blot analysis using anti His_5 antibody. The GST fusion proteins added to the binding reaction mixtures were detected with Ponceau red and shown just below the Western blot analysis, exhibiting almost comparable amount among each fusion protein.

In panel B, yeast two-hybrid analysis was performed between PDZK2, each PDZ domain (PDZ1, PDZ2, PDZ3, PDZ4) or two sequential PDZ domains (PDZ1-2, PDZ2-3, PDZ3-4) of PDZK2 and the C-terminus of OCT/OCTN family or their four amino acid-truncated mutants (OCTN1Δ4 and OCTN2Δ4). Interaction was expressed by growth on agar plates made with medium without histidine (-His). Both T antigen and P53 were used in the control experiment.

In panel C, HEK293/PDZK1, HEK293/PDZK2 and HEK293 cells were transiently transfected with YFP-OCTN2. Cells were solubilized and immunoprecipitated (IP) with c-myc antibody. Western blotting (IB) was then performed using GFP antibody. The total cell lysates were also subjected to SDS-PAGE and their protein amount was checked with Ponceau red; staining was similar in all cell lines (data not shown)..

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Table 1

Inhibitory effects of several compounds on L-[^{3}H]carnitine uptake by HEK293/ PDZK1, HEK293/PDZK2 and HEK293 cells transiently expressing YFP-OCTN2[a)]

Inhibitor	Conc. (mM)	Relative Uptake (% of Control)		
		HEK293/PDZK1	HEK293/PDZK2	HEK293
Control	-	100 ± 2	100 ± 6	100 ± 5
L-carnitine	0.05	7.60 ± 0.24*	9.09 ± 0.46*	14.5 ± 0.6*
L-carnitine	1	0.00 ± 0.25*	1.82 ± 0.49*	4.16 ± 0.70*
TEA	0.5	70.0 ± 0.85*	61.8 ± 3.0*	70.6 ± 2.97*
TEA	1	55.3 ± 0.8*	51.1 ± 2.38*	55.3 ± 2.3*
Verapamil	0.05	7.68 ± 0.92*	11.9 ± 0.7*	18.3 ± 1.5*
Verapamil	1	0.01 ± 0.01*	2.32 ± 0.31*	4.97 ± 3.7*
Procainamide	1	53.3 ± 0.8*	51.1 ± 2.38*	56.1 ± 2.2*
Procainamide	5	23.2 ± 0.9*	21.9 ± 1.0*	24.1 ± 0.9*
NMN	1	85.9 ± 0.7*	81.1 ± 3.5	79.8 ± 3.0*
NMN	5	87.2 ± 0.7*	67.6 ± 3.7*	84.9 ± 3.5

a) HEK293 / PDZK1, HEK293 / PDZK2 and HEK293 cells were transiently transfected with YFP-OCTN2 or pEYFP-C1 vector alone, and uptake of L-[^{3}H]carnitine was then measured for 3 min at 37 ^{o}C in the absence (control) or presence of each compounds. Each value represents the mean ± S.E.M. of three determinations. OCTN2 mediated uptake was shown after subtracting the uptake by mock cells from that by OCTN2, respectively. (n = 3-6)

b) * Significantly different from the control (*$p < 0.01$).

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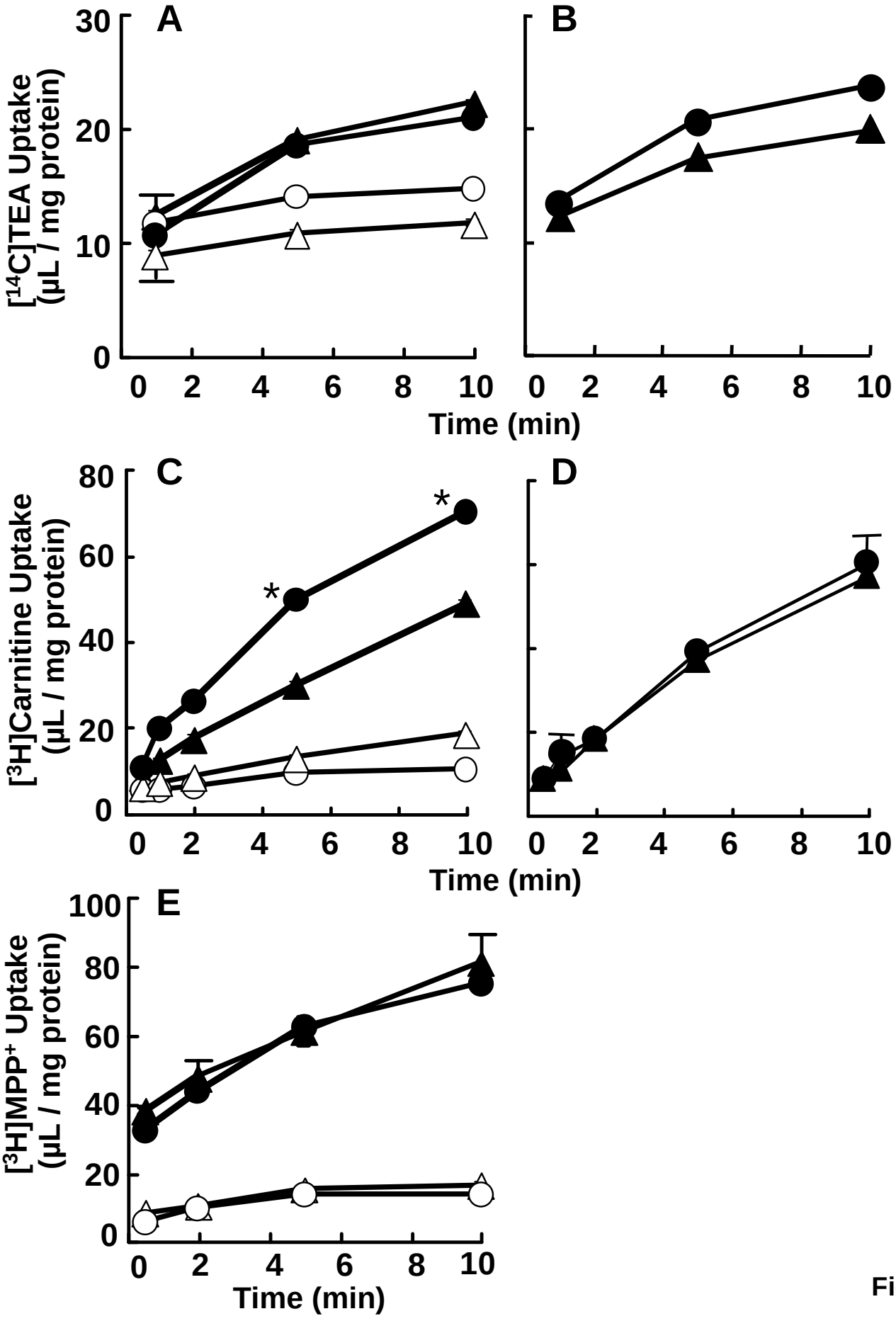

Fig. 1

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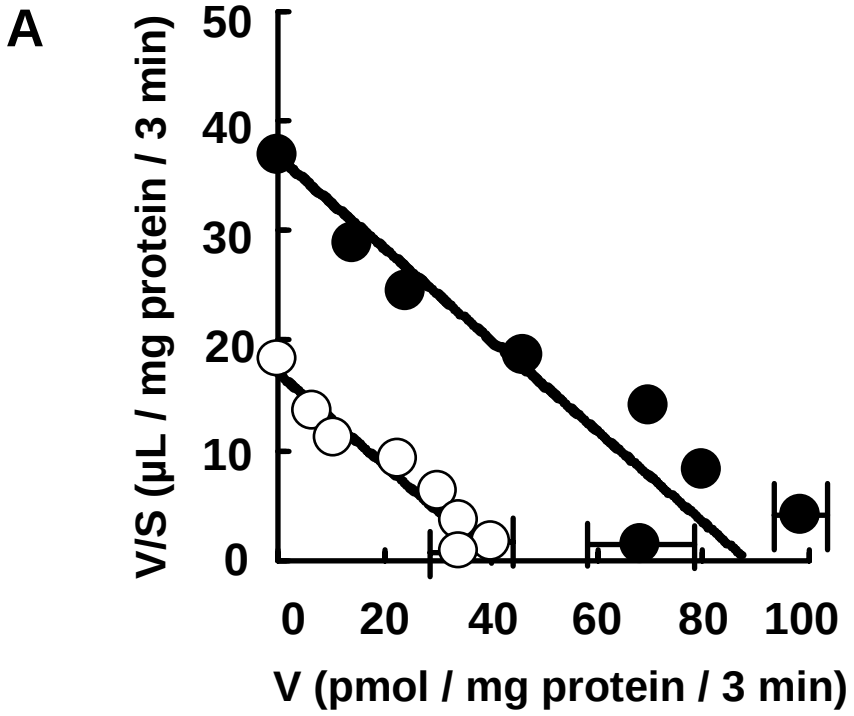

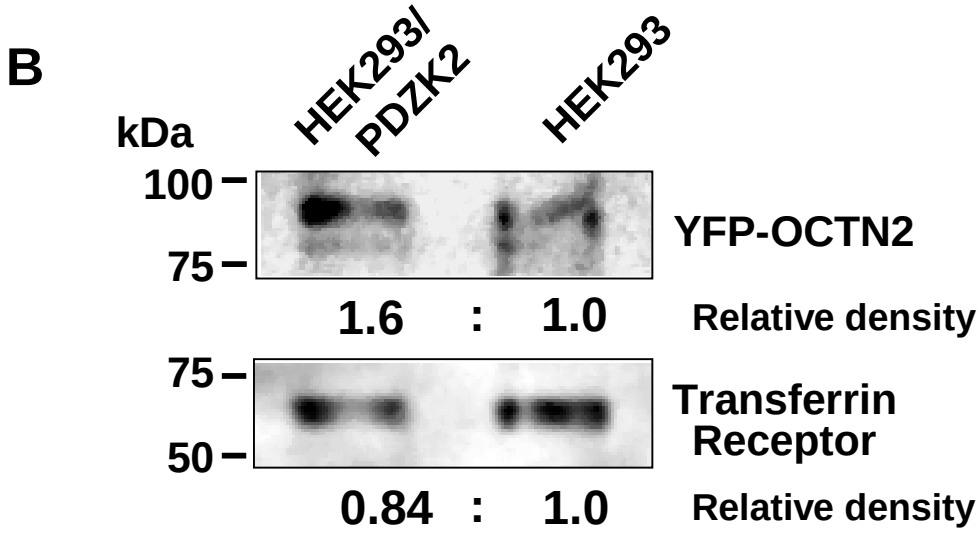

Fig. 2

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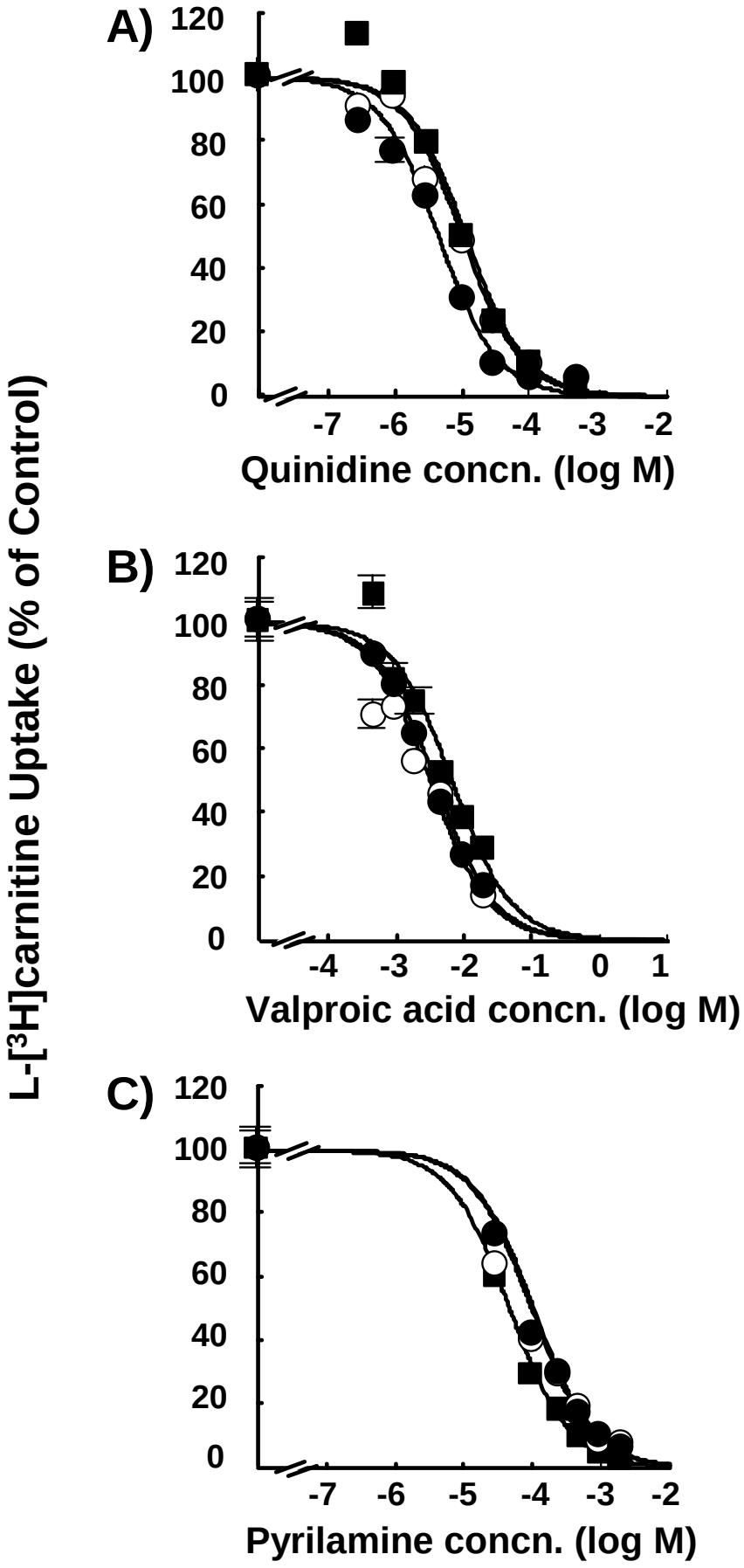

Fig. 3

Fig. 4

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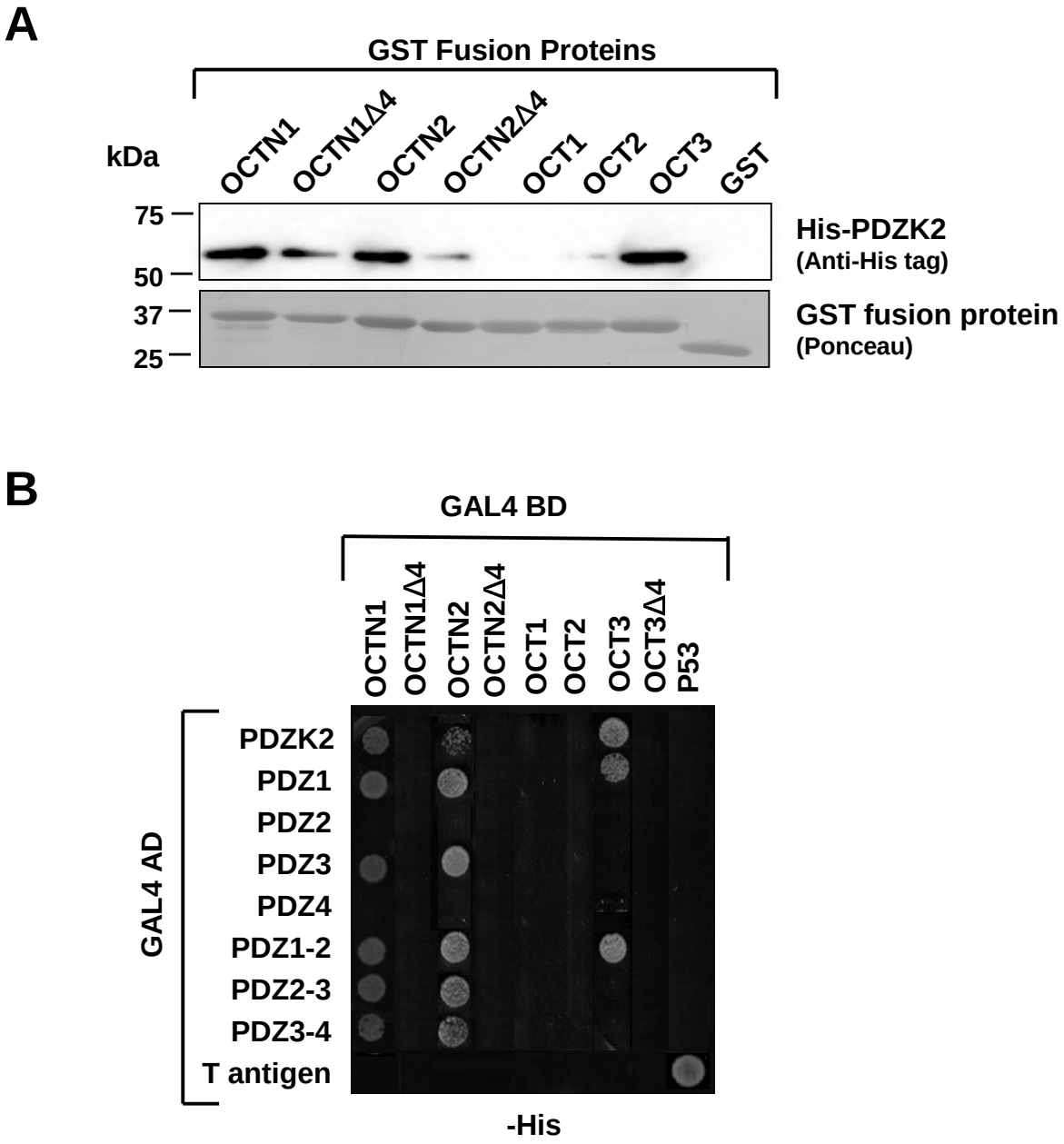

Fig. 5