PDZK1 Regulates Organic Anion Transporting Polypeptide Oatp1a in Mouse Small Intestine

Tomoko Sugiuara1, Toru Otake1, Takuya Shimizu1, Tomohiko Wakahama2, David L. Silver3, Rie Utsumi1, Tomohiro Nishimura1, Shoichi Iseki2, Noritaka Nakamichi1, Yoshiyuki Kubo1, Akira Tsuj1 and Yukio Kato1,*

1Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan
2Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan
3Department of Biochemistry, Albert Einstein College of Medicine, NY, USA

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Summary: Recent studies indicate that various members of the organic anion transporting polypeptide (OATP) family are expressed on apical membranes of the small intestine. In the present study, we investigated possible interaction of Oatp with the PDZ protein PDZK1 in mouse small intestine, using [3H]estrone-3-sulfate (E3S) as a typical substrate. After intraduodenal administration, the level of [3H]E3S appearing in the portal vein of pdzk1 gene knockout (pdzk1−/−) mice was much lower than that in wild-type mice. Lower intestinal absorption of [3H]E3S in pdzk1−/− mice was confirmed in Ussing-type chamber experiments, which showed smaller uptake of [3H]E3S from the apical side in intestinal tissues of pdzk1−/− mice compared with wild-type mice. The kinetics and inhibition profile of [3H]E3S uptake in the Ussing-type chamber were similar to those in HEK293 cells stably expressing Oatp1a5, suggesting involvement of Oatp1a5 in [3H]E3S uptake. Immunoreactivity to anti-Oatp1a antibody was colocalized with PDZK1 in the small intestine of wild-type mice, whereas apical localization of Oatp1a protein was reduced in pdzk1−/− mice. An immunoprecipitation study revealed physical interaction of PDZK1 with Oatp1a. Thus, PDZK1 appears to act as an adaptor for Oatp1a. This is the first demonstration of a regulatory protein directly interacting with small-intestinal OATP.

Keywords: PDZ protein; protein-protein interactions; PDZK1; OATP; SLC transporters; intestinal absorption; estrone-3-sulfate

Introduction

Orally administered drugs are absorbed via intestinal epithelial cells. Carrier-mediated absorption has been proposed to be important for the membrane permeation of drugs ionized in the physiological intestinal lumen, since such drugs are little absorbed by passive diffusion. In particular, carrier-mediated transport through apical membranes of epithelial cells could be the predominant route for overall permeation of organic anions through the intestinal epithelial layers, because cellular uptake of organic anions is electrochemically disadvantageous.

Organic anion transporting polypeptide (OATP) was originally identified as a Na+-independent transporter expressed in the liver. In humans, OATP1A2, OATP1B1, OATP2A1, OATP2B1, OATP3A1 and OATP4A1 have been shown to transport organic anions. Among them, OATP2B1 is expressed on apical membranes of human intestinal epithelial cells and Caco-2 cells, and is a polycpecific transporter for organic anions. OATP1A2 is also present on apical membranes of human small intestine. It has been proposed that decreased oral AUC of fexofenadine when this drug is administered simultaneously with fruit juice is caused by
OATP1A2 inhibition.\textsuperscript{9–11} On the other hand, fexofenadine is recognized not only by OATP1A2,\textsuperscript{12} but also OATP2B1,\textsuperscript{9} and fruit juices inhibit the function of OATP2B1.\textsuperscript{13} Similar inhibition by fruit juices of oral absorption was also reported for several \( \beta \)-blockers,\textsuperscript{14–18} which have recently been identified as substrates of OATP1A2 and OATP2B1.\textsuperscript{19,20} Thus, gastrointestinal absorption of certain therapeutic agents might be mediated at least partly by OATP1A2 and/or OATP2B1 in small intestine.

OATP family orthologs do not exhibit a strict one-to-one relationship between human and rodents. In rodents, there are four Oatp1a molecules, Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1a6, whereas the only ortholog in humans is OATP1A2. On the other hand, Oatp2b1 in rodents corresponds to OATP2B1 in humans. Rodents are most frequently used as experimental animals in preclinical studies, but nevertheless, only limited information is available on the functional expression of Oatp family members in the small intestine of rodents.

Both human OATP1A2 and all mouse Oatp1a orthologs have the amino acid sequence TLK at their extreme C-terminus. This corresponds to the so-called PDZ binding motif, which binds preferentially to class I PDZ domains. Our yeast two-hybrid analysis demonstrated interaction of the C-terminal region of OATP1A2 with at least four PDZ domain-containing proteins (PDZK1, PDZK2, NHERF1 and NHERF2), which are known to bind to other solute carriers (SLC).\textsuperscript{21} Among them, PDZK1 stimulates the substrate uptake activity of certain SLC transporters in double transfectant systems.\textsuperscript{21–23} although the effect of PDZK1 on OATP1A2 has not yet been clarified. Moreover, apical localization of at least two intestinal SLC transporters (oligopeptide transporter, PEPT1; carnitine/organic cation transporter, OCTN2) is reduced in the small intestine of \( p d z k 1^-/- \) mice.\textsuperscript{24} This prompted us to further analyze possible interaction with PDZK1 of small-intestinal Oatp1a using \( p d z k 1^-/- \) mice, with the aim of clarifying the involvement of Oatp1a family members in small-intestinal drug absorption.

In the present study, we examined the roles of PDZK1 in the functions and expression of Oatp1a in the small intestine, using \( p d z k 1^-/- \) mice. To analyze the functions of Oatp1a, gastrointestinal absorption and uptake from the apical side into small-intestinal tissues of estrone-3-sulfate (E3S) was examined, since E3S is a typical substrate of Oatp1a. Polyclonal antibody was also produced in the present study in order to examine the difference in expression and localization of the Oatp1a gene product between wild-type and \( p d z k 1^-/- \) mice. Since functional analysis indicated the possible involvement of Oatp1a5 in small intestinal uptake of E3S, immunoprecipitation and yeast two-hybrid analyses were also performed to examine the interaction between PDZK1 and Oatp1a.

\section*{Materials and Methods}

\textbf{Materials:} [\(^{1}H\)Estrone-3-sulfate ([\(^{1}H\)E3S; 2.12 TBq/mmol] and [\(^{14}C\)Inulin (2.8 mCi/g) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA) and American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), respectively. \([^{14}C]M17055 \) (2.0 GBq/mmol) was synthesized by GE Healthcare (Buckinghamshire, UK). Unlabeled M17055 was produced in the Pharmaceutical Laboratory of Mochida Pharmaceutical Co., Ltd. (Shizuoka, Japan). cDNAs for Oatp1a5 cDNA (BC013694) and Oatp2b1 (BC037089) were purchased from OpenBiosystems (Huntsville, AL) and subcloned into the pcDNA3 vector. All other chemicals were commercial products of analytical grade.

\textbf{Animals:} Male mice were used for all experiments at 6–12 weeks of age. \( p d z k 1^-/- \) mice had been previously produced.\textsuperscript{25} \( p d z k 1^-/- \) and littermates were of a mixed genetic background (C57BL/6J and 129Sv/Ev), produced by intercrossing \( p d z k 1^-/+ \) mice. WKY rats were purchased from Sankyo Labo Service Corporation, Inc. (Toyama, Japan). They had free access to food and water. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Takaramachi Campus of Kanazawa University.

\textbf{Production of polyclonal antibodies:} The 47 and 21 C-terminal amino acids of Oatp1a5 and Oatp2b1, respectively, were cloned by PCR and subcloned into pGE6xP1 vector (GE Healthcare). Recombinant fusion proteins of glutathione S-transferase with the C-terminus were synthesized and injected once a week into female WKY rats as described previously.\textsuperscript{20} Rat polyclonal antibody thus obtained against the C-terminus of Oatp1a5 recognized all Oatp1a subfamily members (Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1a6), but not Oatp2b1, according to Western blot analysis using glutathione S-transferase fusion protein with the C-terminal domains of these transporters. Rat polyclonal antibody against Oatp2b1 recognized Oatp2b1, but not Oatp1a.

\textbf{Uptake study in mouse small intestinal tissues:} The uptake was measured by means of an Ussing-type chamber method as described previously.\textsuperscript{27} The test solution was composed of 128 mM NaCl, 5.1 mM KCl, 1.4 mM CaCl\(_2\), 1.3 mM MgSO\(_4\), 21 mM NaHCO\(_3\), 1.3 mM KH\(_2\)PO\(_4\), 10 mM NaH\(_2\)PO\(_4\), and 5 mM D-glucose [adjusted to pH 6.0 or 7.4 for the apical or basal side, respectively], and gassed with 95% O\(_2\)/5% CO\(_2\) before and during the transport experiment.

\textbf{Transport studies in HEK293 cells stably expressing mouse Oatp1a5 or Oatp2b1:} HEK293 cells were transfected with pcDNA3/Oatp1a5 or pcDNA3/Oatp2b1, and stably transfected cells were selected by adding G418 (Sigma) to the culture medium to obtain HEK293/Oatp1a5 and HEK293/Oatp2b1 cells, respectively. HEK293/Oatp1a5 and HEK293/Oatp2b1
cells were grown in Dulbecco’s modified Eagle’s medium (DMEM #D5796, Sigma, MO) supplemented with 10% FBS, 1% sodium pyruvate (Sigma), 100 unit/mL benzylpenicillin, 0.14 mg/mL streptomycin and 1 mg/mL G418 in a humidified atmosphere of 5% CO₂ in air, in an incubator at 37°C. After reaching confluence, the cells were harvested and suspended in transport buffer containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM H₂PO₄, 1.2 mM MgSO₄, and 25 mM HEPES (adjusted to pH 7.4 with 1 N NaOH). Uptake experiments were then performed using the silicon-oil layer method, as described previously.21

Pharmacokinetic studies: Mice were fasted overnight with free access to water and anesthetized by intraperitoneal injection of pentobarbital. The portal vein was cannulated with silicon tubing (SP10; Natsume, Japan). [³H]E3S (1.5 nmol/kg body weight) dissolved in saline was injected into the intraduodenal space. At various intervals after administration, aliquots of blood were collected through the jugular vein and portal vein. All blood samples were immediately centrifuged to obtain plasma, which was further mixed with Clearsol I (Nacalai Tesque, Inc., Kyoto, Japan) scintillation fluid, and associated radioactivity was measured with a liquid scintillation counter.

The areas under the curve (AUC) of the plasma concentration-time profile of [³H]E3S in the portal vein (AUCpv) and jugular vein (AUCjv) were calculated on the basis of the trapezoidal rule. The fraction of intestinal absorption was calculated as Qp (AUCpv - AUCjv)/Dose, where Qp was portal vein plasma flow rate and assumed to be 0.798 ml/min/kg.28

Immunohistochemical analysis: Frozen sections of mouse small intestine were prepared as described previously.29 For immunofluorescence staining, the sections were first heated to 92°C in Retriever-All™ (Signet Pathology Systems Inc., Dedham, MA). They were then incubated with a mixture of antibodies overnight at 4°C and subsequently incubated with secondary antibodies (Alexa Fluor 594 and Alexa Fluor 488; Invitrogen, Carlsbad, CA) 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). For peroxidase staining, sections were successively pretreated with 0.3% Tween 20 in PBS, 0.3% H₂O₂ in methanol and 5% BSA in PBS and then incubated with anti-Oatp1a or Oatp2b1 antibody. They were then washed with PBS, and the immunoreaction product was visualized by incubating the sections successively with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 2 h, horseradish peroxidase-conjugated streptavidin (DakoCytomation, Kyoto, Japan) for 1 h and 3',3'-diaminobenzidine tetrahydrochloride (DAB) containing H₂O₂ for a few minutes.

Immunoprecipitation study: Small intestinal brush-border membrane vesicles (BBMVs) were prepared from wild-type mice according to a previous report29 and solubilized in RIPA-Y buffer containing 1% Nonidet P-40, 75 mM NaCl, 50 mM Tris-HCl, pH 7.5 and protease inhibitors. Anti-PDZK1 antibody or preimmune serum prebound to Protein L Sepharose (Pierce Chemical, Rockford, IL) was added to the obtained lysate, and the mixture was incubated at 4°C for 8 h, followed by centrifugation and washing three times with PBS. The samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis as described previously.24

Yeast two-hybrid analysis: Clones encoding mouse PDZK1, PDZK2, NHERF1 and NHERF2 were purchased from OpenBiosystems and incorporated into pGADT7(LEU2) vector. The C-terminal 47, 47, 47, 47, 45, 48, 16, 21, 62, 32, 16 and 29 amino acids of Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2, Oatp1c1, Oatp2a1, Oatp2b1, Oatp3a1, Oatp4a1, Oatp6b1, Oatp6c1 and Oatp6d1, respectively, were cloned by PCR and subcloned into pGBK7(LEU) vector. Yeast two-hybrid analysis was performed by cotransfection with pGADT7 and pGBK7 constructs as described previously.21 Co-transformed yeast cells were further cultured on plates lacking leucine, tryptophan and histidine, and the protein-protein interaction was regarded as positive if the yeast cells grew in the medium lacking these three amino acids.

Data analysis: The results were expressed as means ± S.E.M. and statistical analysis was performed using the Student’s t-test. A difference between means was considered significant when p < 0.05. Fitting of plasma concentration-time profiles based on compartmental analysis was performed using the MULTI program.

Results

Small-intestinal absorption of [³H]E3S was reduced in pdzk1−/− mice: To investigate the possible regulation of Oatp1a by PDZK1, intestinal absorption of [³H]E3S, a typical substrate for the Oatp1a family, was first examined in both wild-type and pdzk1−/− mice. To avoid the effect of gastric emptying rate, [³H]E3S was intraduodenally administered. In addition, in view of the rapid disappearance of [³H]E3S due to efficient hepatic uptake,29 we chased portal vein concentration after the administration in order to avoid the hepatic first-pass effect. The time profile of [³H]E3S concentration in circulating plasma was similar in wild-type and pdzk1−/− mice (Fig. 1B). In contrast, portal venous plasma concentration in pdzk1−/− mice was much lower than that in wild-type mice (Fig. 1A). In pdzk1−/− mice, the portal venous concentration was comparable to the circulating plasma concentration (Fig. 1A, 1B), indicating that
**PDZK1 Regulates Oatp1a in Mouse Small Intestine**

![Fig. 1. Plasma concentration-time profiles of [3H]E3S after intraduodenal administration in wild-type and pdzk1−/− mice](Image)

Portal venous (A) and circulating (B) concentrations of [3H]E3S after intraduodenal administration were measured in wild-type (circle) and pdzk1−/− (triangle) mice. Each point represents the mean ± S.E.M. (n = 3–4).

*Significantly different from wild-type mice (p < 0.05).

![Fig. 2. Uptake of [3H]E3S from apical surface of small intestine was reduced in pdzk1−/− mice](Image)

[3H]E3S absorption was minimal.

To investigate the intestinal permeability of [3H]E3S, an uptake study in an Ussing-type chamber was next performed. The uptake of [3H]E3S from the apical surface by the intestinal tissue of wild-type mice was higher than that of pdzk1−/− mice, whereas extracellular space, assessed as the apparent uptake of [14C]inulin, was similar in the two strains (Fig. 2). [3H]E3S uptake in wild-type mice was significantly reduced in the presence of 4 mM unlabeled E3S, whereas such saturation was not clearly observed in pdzk1−/− mice (Fig. 2). The saturable E3S uptake, which was calculated by subtracting [3H]E3S uptake in the presence of unlabeled E3S from that in its absence, was reduced to about 50% in pdzk1−/− mice, compared to that in wild-type mice (Fig. 2).

**Characteristics of [3H]E3S uptake in mouse small intestine:** E3S is a common substrate of various Oatps. Therefore, the interaction of PDZK1 with the C-terminal domain of all the mouse Oatps was next examined in a yeast two-hybrid system (Table 1). PDZK1 and the other

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* Yeast cells were co-transformed with plasmids encoding a full-length mouse PDZ proteins and C-terminal domain of a mouse Oatp family member. Interactions (+) were indicated by the growth of yeast cells on agar plates made with medium lacking histidine. Both T-antigen and p53 were used in the control experiment, showing that there is no non-specific interaction with Oatps and PDZ proteins, respectively.
three PDZ proteins interacted with the Oatp1a subfamily (Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1a6). This is consistent with our previous report that human OATP1A2 interacted with all four PDZ proteins.21) Both Oatp1c1 and Oatp3a1 can also interact with PDZK1, whereas Oatp2b1 did not interact with PDZK1, but interacted only with PDZK2 (Table 1).

mRNAs for both Oatp1a4 and Oatp1a5 were detected in mouse small intestine,31) and the expression of Oatp1a5 protein has been reported in rat.32) Therefore, further studies were performed in Ussing-type chambers to characterize the \( [{}^{3}H] \)E3S uptake from the apical side and to compare its properties with those of Oatp1a5. \( [{}^{3}H] \)E3S uptake by mouse small intestine gradually increased in a time-dependent manner, and the uptake was much higher than that of \( [{}^{14}C] \)inulin (Fig. 3A). The uptake was almost linear for 30 min (Fig. 3A), and therefore, the initial uptake was measured at 30 min. The initial \( [{}^{3}H] \)E3S uptake was next examined in the concentration range of 4.7 nM–4 mM (Fig. 3B). Saturable and non-saturable components were observed (Fig. 3B). \( K_{m} \), \( V_{\text{max}} \) and clearance for the non-saturable component were estimated to be \( 33.9 \pm 20.9 \) \( \mu \)M, \( 1.17 \pm 0.58 \) nmol/cm\(^2\)/30 min and \( 19.1 \pm 1.5 \) \( \mu \)L/cm\(^2\)/30 min, respectively. The initial uptake of \( [{}^{3}H] \)E3S was minimally affected by the replacement of \( \text{Na}^{+} \) with choline (Fig. 3C). In addition, the initial uptake of \( [{}^{3}H] \)E3S was slightly higher at acidic pH compared with that in a neutral condition, whereas \( [{}^{3}H] \)E3S uptake in the presence of 4 mM unlabeled E3S was reduced at all pH values examined (Fig. 3D).

HEK293/Oatp1a5 cells were next established, and the uptake of \( [{}^{3}H] \)E3S was examined. Uptake of \( [{}^{3}H] \)E3S by HEK293/Oatp1a5 was much higher than that by mock cells (Fig. 4A). Uptake of \( [{}^{3}H] \)E3S was saturated only in HEK293/Oatp1a5 cells, but not in mock cells (Fig. 4B). Estimated \( K_{m} \) (15.9 ± 4.4 \( \mu \)M) was close to that found for E3S uptake in mouse small intestine (Fig. 3B).
Fig. 4. Uptake of $[^3]$H]E3S and $[^{14}]$C]M17055 by HEK293/Oatp1a5 and HEK293/Oatp2b1 cells
Time course (A) and concentration dependence (B) of $[^3]$H]E3S (4.2 nM) uptake, and time course of $[^{14}]$C]M17055 uptake (C) were measured in HEK293/Oatp1a5 (○), HEK293/Oatp2b1 (▲) and mock cells (□). In panel B $[^3]$H]E3S has been mixed with various concentrations of unlabeled E3S (0–100 μM). The results are shown as means ± S.E.M. (n = 3–9).

To further characterize the uptake of $[^3]$H]E3S, the inhibitory effects of various compounds on $[^3]$H]E3S uptake by mouse small intestine were examined and compared with that found in HEK293/Oatp1a5 cells. $[^3]$H]E3S uptake by mouse small intestine was reduced in the presence of endogenous hormones, including dehydroepiandrosterone sulfate (DHEAS) and triiodothyronine (T3) (Fig. 5A). Organic anions, including bromosulfophthalein, probenecid, loop diuretics (furosemide and bumetanide), M17055 and some bile acids, inhibited $[^3]$H]E3S uptake by mouse small intestine. On the other hand, OAT inhibitors (p-aminohippuric acid and in-
domethacin and cationic compounds, such as cimetidine and tetraethylammonium, were not inhibitory (Fig. 5A). \[^{[3}H\]E3S uptake by HEK293/Oatp1a5 cells was also inhibited by endogenous hormones, organic anion compounds and bile acids (Fig. 5B), and these inhibition profiles were similar in mouse small intestine and HEK293/Oatp1a5 cells (Fig. 5), suggesting that Oatp1a5 may be, at least in part, responsible for \[^{[3}H\]E3S transport in mouse small intestine. However, both digoxin and indomethacin inhibited \[^{[3}H\]E3S uptake in HEK293/Oatp1a5 cells, but their inhibitory effect in the small intestine was minimal (Fig. 5A, 5B). In addition, the inhibitory effect of bromosulfophthalein, probenecid, M17055 and most of bile salts was not the same for mouse small intestine and HEK293/Oatp1a5 cells (Fig. 5A, 5B). These results suggest possible involvement of multiple transporters in intestinal absorption of E3S.

The uptake of \[^{[3}H\]E3S by HEK293/Oatp2b1 cells was similar to that by mock cells (Fig. 4A), whereas the uptake of \[^{[3}H\]M17055, which is efficiently taken up by human OATP2B1,\(^{33}\) was much higher than that in mock cells (Fig. 4C). Thus, Oatp2b1 seems not to accept E3S as a good substrate.

**Interaction of Oatp1a with PDZK1 in mouse small intestine:**

The localization of Oatp1a and Oatp2b1 in mouse small intestine was then investigated by immunohistochemistry (Fig. 6). Immunostaining with Oatp1a and Oatp2b1 antibodies revealed the presence of Oatp1a and Oatp2b1 in the small intestine of wild-type mice (Fig. 6A, 6D). Pept1, an apical membrane marker, was also detected in the apical region of small intestine (Fig. 6B, 6E). The immunostaining with Oatp1a antibody merged with that with Pept1 antibody (Fig. 6C), whereas immunostaining with Oatp2b1 antibody was only partially colocalized with that of Pept1 antibody (Fig. 6F). These data indicate that Oatp1a is localized on the apical membrane, whereas Oatp2b1 is localized in the subapical region of intestinal epithelial cells.

The expression of Oatp1a and Oatp2b1 was also examined in pdzk1\(^{1/-}\) mice. Immunostaining of Oatp1a was detected on the apical surface of intestinal epithelial cells in wild-type mice (Fig. 7A, arrowhead), but the staining was weak in pdzk1\(^{1/-}\) mice (Fig. 7B). On the other hand, the immunostained image of Oatp2b1 was similar in wild-type and pdzk1\(^{1/-}\) mice (Fig. 7C, 7D). BBMVs were prepared from the small intestine, and Western blot analysis was performed using Oatp1a antibody. An immunoreactive band was detected in BBMVs.
Fig. 7. Interaction of Oatp1a with PDZK1

(A-D) Cryosections of small intestine of wild-type (A, C) and pdzk1−/− (B, D) mice were immunostained with antibodies against Oatp1a (A, B) and Oatp2b1 (C, D). Note that a typical staining pattern of Oatp1a can be observed in apical membrane of small intestine of wild-type mice (A), but the intensity of staining is reduced in pdzk1−/− mice (B). Magnification, ×200. (E) Lysates of small intestinal BBMVs were subjected to SDS-PAGE, followed by Western blot analysis using anti-Oatp1a or β-actin antibody. (F) Lysates of small intestinal BBMVs of wild-type mice were immunoprecipitated with anti-PDZK1 antibody (left panel) or preimmune serum (right panel), followed by Western blot analysis using anti-Oatp1a (upper panel) or anti-PDZK1 (lower panel) antibody.

Prepared from wild-type mice, but minimally found in those from pdzk1−/− mice (Fig. 7E). In contrast, expression of β-actin was observed in both BBMVs (Fig. 7E). These results were compatible with the reduced immunostaining image detected with Oatp1a antibody in pdzk1−/− mice (Fig. 7A, 7B).

Interaction between Oatp1a and PDZK1 was further analyzed by immunoprecipitation. The immunoreaction product obtained in the lysate of BBMVs by anti-PDZK1 antibody was reactive with the anti-Oatp1a antibody, whereas preimmune serum showed no response (Fig. 7F). The immunoreaction product obtained by anti-PDZK1 antibody was reactive with the anti-PDZK1 antibody itself (~81 kD), but that obtained by preimmune serum was not (Fig. 7F), suggesting specific immunoprecipitation with the anti-PDZK1 antibody. Two immunoreactive bands (~76 and 82 kD) were detected by Oatp1a antibody in BBMVs prepared from wild-type mice, and both were reduced in pdzk1−/− mice (Fig. 7E). The size of the larger one was almost the same as that found in the sample immunoprecipitated with anti-PDZK1 antibody (Fig. 7F). The reason for such multiple bands is unknown.
Discussion

The localization of transporters on apical membranes is strictly regulated by scaffold proteins and/or intracellular sorting machinery. Such regulatory mechanism(s) are probably required for efficiency and fidelity in the vectorial transport of endogenous solutes, nutrients and xenobiotics. Several OATPs are known to be expressed in the small intestine and could be involved in gastrointestinal absorption of certain types of therapeutic agents. Nevertheless, no information is available on proteins that interact directly with small-intestinal OATPs. PDZK1 is one of the scaffold proteins which affect subcellular localization, expression or functions of various transporters. PDZK1 interacts in vitro with the C-terminal domain of at least the OATP1A family in both humans and mice (Table 1). PDZK1 is mainly localized in microvilli of the apical region in small-intestinal absorptive cells. In pdzk1−/− mice, expression of several SLC transporters was decreased at the apical membrane of intestinal epithelial cells.

In the present study, Oatp1a was localized on apical membranes of intestinal epithelial cells in wild-type mice (Fig. 6A–C, 7A), but this apical localization was greatly reduced in pdzk1−/− mice (Fig. 1). This was compatible with the decrease in E3S uptake from the apical surface assessed in Ussing-type chambers (Fig. 2). The reduction in the absorption after intraduodenal administration of the Oatp1a substrate E3S was similar between wild-type and pdzk1−/− mice (Fig. 1A, 1B). PDZK1 interacts with Oatp1a with PDZK1 (Fig. 7F). Finally, gastrointestinal absorption of the Oatp1a substrate E3S after intraduodenal administration was reduced in pdzk1−/− mice (Fig. 1). This was consistent with that of cephalaxin (PEPT1 substrate) and carnitine (OCTN2 substrate) in pdzk1−/− mice. In contrast, immunostaining of Oatp2b1, which does not interact with PDZK1 (Table 1), was similar between wild-type and pdzk1−/− mice (Fig. 7C, D). Thus, PDZK1 interacts with Oatp1a in the small intestine in vivo, and this interaction could be important for the localization of this transporter on apical membranes of epithelial cells.

Gene deletion technology can be applied to knock out transporters in mice. Therefore, mice have been widely used to identify transporter molecules involved in membrane permeability. Nevertheless, it has not yet been clarified which Oatp transporters are functionally expressed in the small intestine in mice. The present results indicate that Oatp1a5 is at least partially involved in [3H]E3S uptake from the apical surface in mouse small intestine, based on the similarity in Km value (Fig. 3B, 4B) and inhibition profile (Fig. 5) for [3H]E3S uptake between mouse small-intestinal tissue and HEK293/Oatp1a5 cells. The values of uptake clearance (Vmax/Km) of the saturable and nonsaturable components were estimated to be 34.5 and 19.1 µL/cm²/30 min (Fig. 3B), respectively, indicating that the saturable component accounts for ~64% of total uptake of [3H]E3S in the small intestine. However, inhibitory effects of several compounds were not the same between the small intestine and HEK293/Oatp1a5 cells (Fig. 5A, 5B), suggesting that one or more transporters are involved in the intestinal absorption of E3S. Cheng et al. reported the tissue distribution of mRNAs for mouse Oatp family members, indicating that Oatp1a4 and Oatp1a5, both of which transport E3S are present in the small intestine. However, Cheng et al. also reported that Oatp2b1 shows a higher mRNA level than other Oatp family members in the small intestine. However, Oatp2b1 only minimally transported E3S (Fig. 4A), although E3S is a good substrate of human OATP2B1. Expression of the gene product has also been reported for Oatp1a5 in rodents, although the precise identification of the Oatp1a subfamily members has not yet been performed. According to these findings, mouse Oatp2b1 has some differences in substrate specificity from human OATP2B1, and the contribution of Oatp2b1 to the small-intestinal uptake of E3S could be minor.

In the present study, appearance of [3H]E3S in the systemic circulation after intraduodenal administration was similar between wild-type and pdzk1−/− mice (Fig. 1B), and the only difference between the two strains was in portal venous concentration (Fig. 1A). This is in contrast to our previous observation that appearance of both cephalaxin and carnitine in the systemic circulation was reduced in pdzk1−/− mice compared with wild-type mice. The fraction of intestinal absorption of [3H]E3S was calculated from the difference between the portal vein and systemic concentrations as 14.5% and 0.5% in wild-type and pdzk1−/− mice, respectively, in the present study. This result is consistent with the reduction of intestinal uptake from the apical side in pdzk1−/− mice (Fig. 2) and the reduction of Oatp1a from the apical surface in pdzk1−/− mice (Fig. 7B). Wang et al. reported that Oatp1a1 is internalized in hepatocytes of pdzk1−/− mice, and consequently plasma disappearance of bromosulphthalein, a substrate of Oatp1a1, is delayed in pdzk1−/− mice. E3S is also a substrate of Oatp1a1 and is efficiently taken up by hepatocytes. Therefore, it is possible that the hepatic first-pass effect for E3S may partially compensate for the phenotype observed in the systemic circulation (Fig. 1B). This would be in contrast to the pharmacokinetic properties of cephalaxin and carnitine, which both show quite minor hepatic first-pass effect. OATPs are expressed not only on apical membranes of small intestine, but also on sinusoidal membrane of hepatocytes. OATPs in the liver may facilitate hepatic uptake of various drugs including statins, fexofenadine, repaglinide. While the OATP family members present in liver and small intestine are mostly different, these OATPs have partially overlapping substrate speci-
ficity. Therefore, as in the case of deficiency of PDZK1, concomitant knockdown (for example by drug interaction) of both hepatic and intestinal OATPs may also result in compensation in the systemic exposure, leading to minimal change of drug concentration in the circulation. Nevertheless, even if the circulating drug concentration is unchanged, it should be noted that both intestinal absorption and hepatic drug exposure may be reduced in such circumstances.

The physiological significance of the interaction between PDZK1 and Oatp1a should be clarified by further analyses, although coupling with other transporters and/or membrane proteins could be one of the possible functions of PDZK1. PDZ proteins including PDZKs and NHERFs have four and two PDZ domains in their structure, respectively, and each PDZ domain interacts with membrane proteins and/or transporters. The intestinal anion exchanger DRA (downregulated in adenoma) is, for example, coupled with CFTR and NHE3 in the upper part of the small intestine. The C-terminus of various transporters may interact with C-terminal of PDZK1. The intestinal anion exchange DRA is downregulated in adenoma and its expression is unchanged, it should be noted that both intestinal absorption and hepatic drug exposure may be reduced in such circumstances.

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