

# Organic Cation/Carnitine Transporter OCTN2 (Slc22a5) Is Responsible for Carnitine Transport across Apical Membranes of Small Intestinal Epithelial Cells in Mouse

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## ABSTRACT

The organic cation/carnitine transporter OCTN2 is responsible for renal tubular reabsorption of its endogenous substrate, carnitine, although its physiological role in small intestine remains controversial. Here we present direct evidence for a predominant role of OCTN2 in small intestinal absorption of carnitine based on experiments with juvenile visceral steatosis (*jvs*) mice, which have a hereditary deficiency of the *octn2* gene. Uptake of carnitine, assessed with an Ussing-type chamber system, from the apical surface of the small intestine was saturable and higher than that from the basal surface in wild-type mice, whereas carnitine uptake having these characteristics was almost absent in *jvs* mice. Saturable uptake of carnitine was also confirmed in isolated enterocytes obtained from wild-type mice, and the  $K_m$  value obtained ( $\sim 20 \mu\text{M}$ ) was close to that reported for carnitine uptake by human embryonic kid-

ney 293 cells stably expressing mouse OCTN2 (Slc22a5). The carnitine uptake by enterocytes was decreased in the presence of various types of organic cations, and this inhibition profile was similar to that of mouse OCTN2, whereas uptake of carnitine was quite small and unsaturable in enterocytes obtained from *jvs* mice. Immunohistochemical and immunoprecipitation analyses suggested colocalization of OCTN2 with PDZK1, an adaptor protein that functionally regulates OCTN2. Immunoelectron microscopy visualized both OCTN2 and PDZK1 in microvilli of absorptive epithelial cells. These findings indicate that OCTN2 is predominantly responsible for the uptake of carnitine from the apical surface of mouse small intestinal epithelial cells, and it may therefore be a promising target for oral delivery of therapeutic agents that are OCTN2 substrates.

Organic cation/carnitine transporters (OCTNs) belong to the solute carrier (SLC) 22A family, which also includes organic anion transporters and organic cation transporters. Human OCTN1 (SLC22A4), which was first cloned from fetal liver, transports various organic cations (Tamai et al., 1997; Yabuuchi et al., 1999) while showing high transport activity for ergothioneine and stachydrine, which are zwitterionic compounds (Grundemann et al., 2005). The second member of the OCTN family in humans, OCTN2 (SLC22A5), also transports various types of organic cations (Tamai et al.,

1998; Wu et al., 1998; Ohashi et al., 1999). Both OCTN1 and OCTN2 in humans and mice  $\text{Na}^+$ -dependently transport carnitine, a zwitterionic compound that is essential for the  $\beta$ -oxidation of fatty acids in mitochondria. In mouse kidney, OCTN2 (Slc22a5) is localized in apical membranes of proximal tubules (Tamai et al., 2001, 2004). Mutation in the *OCTN2* gene in humans causes systemic carnitine deficiency (SCD) caused by an increase in urinary excretion of carnitine (Nezu et al., 1999). Thus, OCTN2 in kidney has been characterized as a homeostatic regulator of its endogenous substrate, carnitine.

Involvement of OCTN2 in small intestinal carnitine transport is still controversial. Shaw et al. (1983) and Hamilton et al. (1986) have characterized the uptake mechanism for carnitine in small intestine and reported saturable and  $\text{Na}^+$ -dependent carnitine transport in rat everted intestinal sacs and human intestinal biopsy specimens. Gudjonsson et al.

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**ABBREVIATIONS:** OCTN2, organic cation/carnitine transporter 2; *Jvs*, juvenile visceral steatosis; SLC, solute carrier; SCD, systemic carnitine deficiency; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; MES, 4-morpholineethanesulfonic acid.

(1985) also demonstrated saturable carnitine transport at the rat intestinal apical membrane using a perfusion technique. However, the values of the Michaelis constant ( $K_m$ ) for carnitine transport observed in these studies (200–300  $\mu\text{M}$ , Shaw et al., 1983; ~560  $\mu\text{M}$ , Hamilton et al., 1986; 1–1.3 mM, Gudjonsson et al., 1985) are much higher than those reported for human, rat, and mouse OCTN2 (4–20  $\mu\text{M}$ ) (Tamai et al., 1998, 2000; Wu et al., 1999). Duran et al. (2002) reported that uptake of carnitine by brush-border membrane vesicles obtained from chicken small intestine is  $\text{Na}^+$ -dependent, membrane voltage-dependent, and pH-dependent, with a  $K_m$  value of 26 to 31  $\mu\text{M}$ . They detected OCTN2 mRNA expression in intestinal villus (Duran et al., 2002). Duran et al. (2005) recently demonstrated immunoreactive localization of OCTN2 and OCTN3 (a third member of the OCTN family in mouse) on apical and basolateral membranes, respectively, of rat and chicken enterocytes. On the other hand, Li et al. (1990) suggested a predominant role of passive diffusion in carnitine transport at brush-border membranes in rats. Lahjouji et al. (2002) reported that uptake of carnitine by basolateral membrane vesicles obtained from mouse intestine is  $\text{Na}^+$ -dependent, whereas that by brush-border membrane vesicles is  $\text{Na}^+$ -independent, although the OCTN2 gene product was not detected in either case. Most of these data suggest a possible involvement of OCTN2 in carnitine transport in small intestine, although the molecular mechanism(s) has not yet been established.

The juvenile visceral steatosis (*jvs*) mouse, which has a hereditary deficiency of the *octn2* gene, is a promising tool to clarify the pharmacological roles of OCTN2 (Nezu et al., 1999). The *jvs* mouse exhibits SCD caused by increased urinary excretion of carnitine, as is seen in human patients with SCD. Yokogawa et al. (1999) reported reduced oral bioavailability of carnitine in *jvs* mice compared with wild-type mice, suggesting the possible involvement of OCTN2 in the gastrointestinal absorption of carnitine, although functional expression of OCTN2 in the small intestine had not been established. Considering that the appropriate systemic carnitine level is maintained by gastrointestinal absorption from the diet and by a modest rate of biosynthesis and efficient renal reabsorption (Rebouche, 2004), it is important to clarify the major mechanisms involved in carnitine transport across intestinal epithelial cells to understand carnitine homeostasis. In addition, gastrointestinal absorption of certain types of therapeutic agents has been suggested to be mediated by transporters that are expressed on apical membranes of intestinal epithelial cells (Tsuji et al., 1977; Han and Amidon, 2000; Kunta and Sinko, 2004; Sai and Tsuji, 2004). Because these transporters could also be involved in the absorption of nutrients and hormones, identification of the carnitine transport system in small intestine could provide another good candidate target for oral drug delivery. In the present study, we attempted to demonstrate directly a fundamental role of OCTN2 in carnitine transport across apical membranes of small intestinal epithelial cells by using *jvs* mice. In addition, to understand the possible regulatory mechanisms for OCTN2-mediated carnitine transport, we also examined the protein-protein interaction of OCTN2 in small intestine with PDZK1, which directly stimulates the transport function of OCTN2 (Kato et al., 2005).

## Materials and Methods

**Materials.** Rabbit polyclonal antibodies for mouse OCTN1 and OCTN2 and rat polyclonal antibody for PDZK1 were raised as described previously (Tamai et al., 2000; Kato et al., 2005). Monoclonal antibodies against  $\text{Na}^+/\text{K}^+$  ATPase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Upstate Biotechnology (Lake Placid, NY) and CHEMICON International Inc. (Temecula, CA), respectively. L-[ $^3\text{H}$ ]Carnitine (3.1 TBq/mmol) was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). [ $^{14}\text{C}$ ]Inulin (89 MBq/g) was from PerkinElmer Life Sciences (Boston, MA). Hyaluronidase (bovine testis Type 1-S) was purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from Sigma Chemical, Wako Pure Chemical Industries (Osaka, Japan), Funakoshi Co. (Tokyo, Japan), and Nacalai Tesque, Inc. (Kyoto, Japan) and used without further purification.

**Animals.** Homozygous mutant mice (*jvs*) designated as *jvs/jvs* were identified as those having a swollen, fatty liver by observation through the abdominal wall at 2 to 5 days after birth and confirmed by genotyping of the litter mates. By mating heterozygous male mice with heterozygous female mice, we obtained three genetic types of the mice: homozygous mutants (*jvs/jvs*), heterozygous *jvs* mice (+/*jvs*), and wild type (+/+). Because of SCD in *jvs* mice, they were routinely provided with a high-carnitine diet that contained 0.1% carnitine (Ninox Lab Supply Inc., Kahoku-gun, Japan). Male C3H.OH mice (6–8 weeks old) were purchased from Sankyo Labo Service Corporation, Inc. (Toyama, Japan). This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

**Preparation of Isolated Enterocytes.** Cell isolation was carried out by using hyaluronidase to enzymatically release enterocytes from mouse small intestine according to the method described by Kimmich (1970). After incubation of small intestinal tissues in the presence of suspension buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 5.6 mM D-glucose, 1.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , and 25 mM HEPES at pH 7.0) that also contained 1.5 mg/ml hyaluronidase and 1 mg/ml bovine serum albumin for 40 min, cells were released from the intestine by gentle agitation and washed twice by centrifugation at 100g for 1 min with transport buffer that contained the same solutes as the suspension buffer, except that 25 mM MES, pH 6.0, was used instead of HEPES.

**Transport Studies in Isolated Enterocytes.** Isolated enterocytes were incubated in the transport buffer containing L-[ $^3\text{H}$ ]carnitine. At the designated times, 200- $\mu\text{l}$  aliquots of the mixture were withdrawn, and the enterocytes 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) with a density of 1.015 on top of 3 M KOH solution. After solubilization of each enterocyte pellet in KOH, the enterocyte lysate was neutralized with HCl. The associated radioactivity was measured with a liquid scintillation counter, LSC-5100 (Aloka, Tokyo, Japan) with Clearsolv I (Nacalai Tesque, Inc., Kyoto, Japan) as a scintillation fluid. Cellular protein content was determined using the Bradford method with a protein assay kit (Bio-Rad, Hercules, CA). Kinetic parameters for transport activity were estimated by nonlinear least-squares fitting of the data to the following equation using the MULTI program:

$$V = V_{\max}S/(K_m + S) + K_{ns} \cdot S$$

where  $V$ ,  $S$ ,  $K_m$ ,  $V_{\max}$ , and  $K_{ns}$  represent uptake velocity, substrate concentration, Michaelis constant, maximum uptake velocity, and intrinsic clearance for nonsaturable uptake, respectively.

**Uptake Studies in an Ussing-Type Chamber.** Mice intestinal tissue sheets were prepared as described previously (Naruhashi et al., 2002). The tissue preparation, consisting of the mucosa and most of the muscularis mucosa, was made by removing the submucosa and tunica muscularis with fine forceps. The tissue sheets were mounted

vertically in an Ussing-type chamber that provided an exposed area of 0.25 cm<sup>2</sup>. Transport buffer (128 mM NaCl, 5.1 mM KCl, 1.3 mM KH<sub>2</sub>PO<sub>4</sub>, 5.0 mM D-glucose, 1.4 mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub>, 21 mM NaHCO<sub>3</sub>, and 10 mM NaH<sub>2</sub>PO<sub>4</sub>) at pH values of 7.4 and 6.0 was used as a bathing solution at the basal and apical sides, respectively. The volume of the bathing solution on each side was 1.2 ml, and the temperature was maintained at 37°C in a water-jacketed reservoir. To examine the Na<sup>+</sup> dependence of uptake, Na<sup>+</sup> was replaced with choline in the bathing solution. At the designated times, tissue samples were washed with ice-cold buffer and then solubilized in 1 ml of Soluene-350 (Packard Co., Canberra, Australia) by incubation at room temperature for 24 h. The solubilized samples were neutralized with HCl, left at room temperature for 4 h, and mixed with 3 ml of scintillation cocktail. The radioactivity was counted in a liquid scintillation counter.

**Immunofluorescent Microscopy.** Frozen sections of mouse small intestines were prepared as described previously (Tamai et al., 2004). The formaldehyde-fixed sections were heated to 92°C in RETRIEVE-ALL (SIGENT Pathology Systems Inc., Dedham, MA) buffer. They were then incubated with a mixture of antibodies for 1 h and further incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG conjugate, Alexa Fluor 488 goat anti-rat IgG conjugate, and Alexa Fluor 594 anti-rabbit IgG conjugate) (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 samples. The specimens were examined with an Axiovert S 100 microscope (Carl Zeiss, Jena, Germany) and a confocal laser scanning fluorescence microscope (LSM 510; Carl Zeiss).

**Electron Microscopic Analysis of Localization of OCTN2 and PDZK1.** For electron microscopic analysis, the pre-embedding immunoreaction method was used (Wakayama et al., 2004). The cryosections immunostained with anti-OCTN2 or anti-PDZK1 antibodies were postfixed in 0.5% OsO<sub>4</sub> for 20 min, stained with 1% uranyl acetate for 20 min, dehydrated in graded ethanol series, and embedded in Glicidether 100 (Selva Fenbiochemica, Heidelberg, Germany). Ultrathin sections were cut with an ultramicrotome and observed with a Hitachi H-700 electron microscope (Hitachi, Yokohama, Japan).

**Immunoprecipitation and Western Blot Analyses in Small Intestinal Mucosa.** Small intestinal mucosa was obtained from three male mice, homogenized, 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 prebound to Protein L Sepharose (Pierce Chemical, Rockford, IL) or anti-GAPDH antibody prebound to Protein G Sepharose (Amersham) was then added to the obtained lysate, and the mixture was incubated at 4°C for 8 h, followed by centrifugation and washing three times with phosphate-buffered saline. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-OCTN2, anti-PDZK1, or anti-GAPDH antibodies. Western blot analysis for expression of OCTN2 in small intestinal mucosa was performed as described above.

## Results

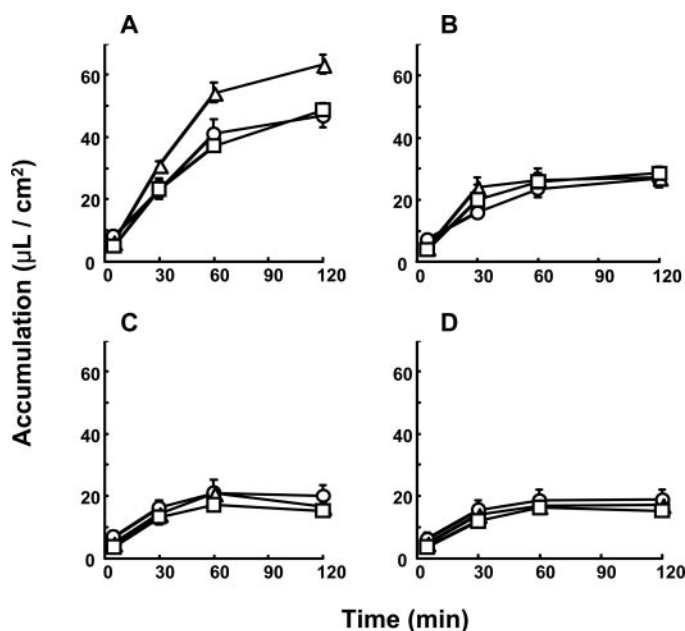
**Characterization of Carnitine Uptake in Small Intestines of Wild-Type and *jvs* Mice.** To characterize the uptake mechanism(s) of carnitine in small intestine, uptakes of [<sup>3</sup>H]carnitine from the apical and basal sides of small intestine were first examined in an Ussing-type chamber (Fig. 1). In wild-type mice, uptake of [<sup>3</sup>H]carnitine from the apical side was much higher than that from the basal side (Fig. 1, A and B). The apical uptake of [<sup>3</sup>H]carnitine was higher in the middle part of the small intestine, compared with the upper and lower parts (Fig. 1A). To examine possible involvement of OCTN2, we used *jvs* mice, which have a hereditary deficiency

of the *octn2* gene. The [<sup>3</sup>H]carnitine uptake from the apical side of the small intestine of *jvs* mice was much lower than that in wild-type mice (Fig. 1, A and C). In *jvs* mice, uptakes of [<sup>3</sup>H]carnitine at the apical and basal sides of the small intestine were comparable (Fig. 1, C and D), and the apical carnitine uptake showed little variation among the upper, middle, and lower parts of the small intestine (Fig. 1C).

Uptake of [<sup>3</sup>H]carnitine from the apical side of the small intestine in wild-type mice was reduced in the presence of 20 mM concentration of unlabeled carnitine (Fig. 2, A, C, and E), whereas there was little decrease in *jvs* mice (Fig. 2, B, D, and F). In addition, the uptake of [<sup>3</sup>H]carnitine from the apical side in wild-type mice was decreased when Na<sup>+</sup> in the medium was replaced with choline (Fig. 2A), whereas such an effect of Na<sup>+</sup> replacement in *jvs* mice was minimal (Fig. 2B).

Uptake of [<sup>14</sup>C]inulin, an extracellular marker, was also examined using the Ussing-type chamber method and was found to amount to 2.84 ± 0.06 and 2.56 ± 0.08 μl/cm<sup>2</sup> after 120-min incubation in the middle part of the small intestine from wild-type and *jvs* mice, respectively (mean ± S.E.M. of three animals).

**Characterization of Carnitine Uptake in Enterocytes Isolated from Wild-Type and *jvs* Mice.** To characterize further the uptake mechanism of carnitine, isolated enterocytes were prepared both from wild-type and *jvs* mice, and uptake of carnitine was measured. Uptake of [<sup>3</sup>H]carnitine in enterocytes obtained from wild-type mice was much higher than that in *jvs* mice (Fig. 3). Uptake of [<sup>3</sup>H]carnitine in enterocytes of wild-type mice was decreased in the presence of 20 mM concentration of unlabeled carnitine or when Na<sup>+</sup> was replaced with choline (Fig. 3A), whereas such effects were minimal in the case of uptake by enterocytes of *jvs*



**Fig. 1.** Time course of carnitine uptake at the small intestine in wild-type and *jvs* mice. Uptakes of [<sup>3</sup>H]carnitine (12 nM) from the apical (A and B) and basal (C and D) sides of the small intestine of wild-type (A and C) and *jvs* (B and D) mice were measured in an Ussing-type chamber system in the presence of Na<sup>+</sup>. Intestinal tissues were obtained from the upper (○), middle (Δ), and lower (□) parts of the intestine of each mouse strain. Data were normalized to medium substrate concentration and are mean values ± S.E.M. (n = 3).



mice (Fig. 3B). The extracellular space assessed in terms of [ $^{14}$ C]inulin uptake for 3 min was  $2.84 \pm 0.06 \mu\text{L}/\text{mg}$  protein (mean  $\pm$  S.E.M. of three animals).

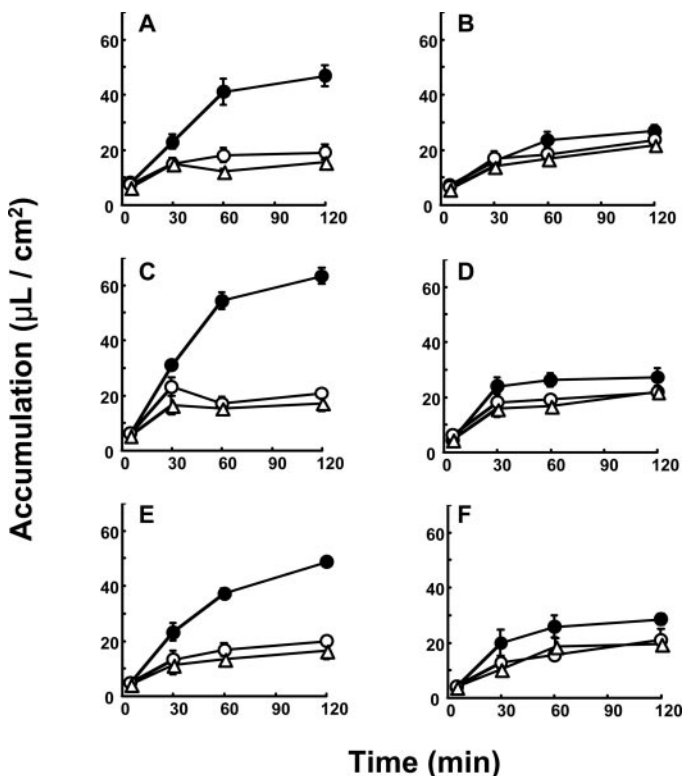
Kinetic analysis of carnitine uptake was then performed in enterocytes of both wild-type and *jvs* mice (Fig. 4). Uptake of carnitine in wild-type mice exhibited saturable and nonsaturable components with  $K_m$ ,  $V_{\max}$ , and  $K_{ns}$  values of  $12.0 \pm 2.9 \mu\text{M}$ ,  $80.7 \pm 14.2 \text{ pmol}/\text{mg}$  of protein/3 min, and  $1.18 \pm 0.11 \mu\text{L}/\text{mg}$  of protein/3 min, respectively (Fig. 4A). The in-

trinsic clearance for the saturable component ( $V_{\max}/K_m$ ) was approximately 6 times higher than  $K_{ns}$ , suggesting that the carnitine uptake is mainly governed by the saturable component. On the other hand, uptake of carnitine in *jvs* mice exhibited only a nonsaturable component, with  $K_{ns}$  of  $2.06 \pm 0.05 \mu\text{L}/\text{mg}$  of protein/3 min (Fig. 4B).

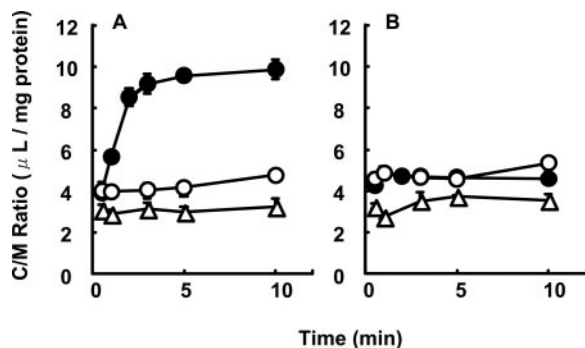
To evaluate further the functional similarity between the intestinal carnitine transport system and OCTN2, the inhibitory effects of various compounds, most of which are substrates and/or inhibitors of mouse OCTN2-mediated carnitine transport, on carnitine transport by the enterocytes were examined next (Table 1). The uptake of carnitine by enterocytes of wild-type mice was significantly decreased in the presence of various compounds, including D-carnitine, acetyl-L-carnitine, acetylcholine, dopamine, thiamine, quinidine, verapamil, tetraethylammonium, 1-methyl-4-phenylpyridinium, and pyrilamine (Table 1). Significant inhibition was not observed with serotonin, norepinephrine, histamine,  $\alpha$ -ketoglutarate, guanidine,  $N^1$ -methylnicotinamide, diphenhydramine, procainamide, lidocaine, or *para*-aminohippurate (Table 1). This inhibition profile is similar to that observed for carnitine uptake by HEK293 cells stably transfected with mouse OCTN2 (Tamai et al., 2000; Ohashi et al., 2001), except that 5 mM norepinephrine and 500  $\mu\text{M}$  diphenhydramine, procainamide, and lidocaine significantly inhibited mouse OCTN2-mediated carnitine uptake (Ohashi et al., 2001), whereas such inhibition was not observed in enterocytes (Table 1).

Because we routinely breed *jvs* mice using a high-carnitine diet, similar uptake studies using enterocytes isolated from *jvs* mice maintained on a normal diet after weaning were performed to rule out any possible effect of dietary carnitine on carnitine transport in the small intestine. Uptake of [ $^3\text{H}$ ]carnitine in *jvs* mouse (6–8 weeks old) maintained on a normal diet was similar to that in *jvs* mice given the high-carnitine diet, suggesting that carnitine in the diet has little effect on the intestinal carnitine transport characteristics in *jvs* mice.

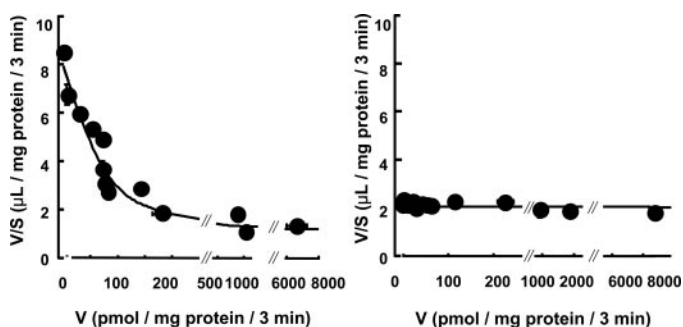
**OCTN2 Is Colocalized with PDZK1 in the Brush-Border of Apical Membranes.** Next, to examine the localization of OCTN2 in mouse small intestine, cryosections were stained with polyclonal antibodies against OCTN2 (Fig. 5). Immunoreactive fluorescence was detected mainly on apical membranes of intestinal absorptive cells, and this was in contrast with the basolateral staining seen with  $\text{Na}^+/\text{K}^+$  ATPase antibody (Fig. 5, A–C). To support the localization of



**Fig. 2.** Effect of unlabeled carnitine and replacement of  $\text{Na}^+$  on uptake of [ $^3\text{H}$ ]carnitine from the apical side of the small intestine. [ $^3\text{H}$ ]Carnitine (12 nM) alone in the absence ( $\Delta$ ) or presence ( $\bullet$ ) of  $\text{Na}^+$  or [ $^3\text{H}$ ]carnitine with 20 mM unlabeled carnitine in the presence of  $\text{Na}^+$  ( $\circ$ ) was added to the apical side of an Ussing-type chamber, and uptakes of [ $^3\text{H}$ ]carnitine at the upper (A and B), middle (C and D), and lower (E and F) parts of the small intestine obtained from wild-type (A, C, and E) and *jvs* (B, D, and F) mice were measured. Data were normalized to medium substrate concentration and are mean values  $\pm$  S.E.M. ( $n = 3$ ).



**Fig. 3.** Time course of carnitine uptake by isolated mouse enterocytes. Isolated enterocytes obtained from wild-type (A) and *jvs* (B) mice were incubated with [ $^3\text{H}$ ]carnitine (12 nM) alone in the absence ( $\Delta$ ) or presence ( $\bullet$ ) of  $\text{Na}^+$  or [ $^3\text{H}$ ]carnitine with 20 mM unlabeled carnitine ( $\circ$ ) in the presence of  $\text{Na}^+$  at  $37^\circ\text{C}$ . Uptake of [ $^3\text{H}$ ]carnitine was then measured by the silicon-layer method. Data were normalized to medium substrate concentration and are mean values  $\pm$  S.E.M. ( $n = 3$ ).



**Fig. 4.** Kinetic analysis of carnitine uptake by isolated mouse enterocytes. Isolated enterocytes obtained from wild-type (A) and *jvs* (B) mice were incubated for 3 min with various concentrations of [ $^3\text{H}$ ]carnitine at  $37^\circ\text{C}$ , and uptake of [ $^3\text{H}$ ]carnitine was measured. Uptake of [ $^{14}\text{C}$ ]inulin was then subtracted from the [ $^3\text{H}$ ]carnitine uptake. Data were obtained from three independent preparations and are shown as Eadie-Hofstee plots (mean  $\pm$  S.E.M.).

**Expression and Physical Interaction of OCTN2 with PDZK1 in Mouse Small Intestine.** To estimate the expression level of OCTN2 in each segment of mouse small intestine, Western blot analysis was performed with intestinal mucosal fractions. An immunoreactive band of approxi-

PDZK1 directly stimulates the transport function of human OCTN2, at least in *in vitro* cell lines (Kato et al., 2005), and might therefore be involved in carnitine absorption mechanisms in the small intestine. To examine the direct interaction of the two proteins, immunoprecipitation of lysates of small intestinal mucosa was performed using anti-PDZK1 antibody followed by Western blot analysis with anti-OCTN2 antibody (Fig. 7B). A band immunoreactive with anti-OCTN2 antibody was observed after immunoprecipitation with anti-PDZK1 antibody but not when preimmune serum or anti-GAPDH antibody was used in place of anti-PDZK1 antibody (Fig. 7B). The immunoprecipitate was immunoreactive with anti-PDZK1 antibody itself (Fig. 7B). The immunoprecipitate with anti-GAPDH antibody was also immunoreactive with anti-GAPDH antibody itself (Fig. 7B).

Carnitine homeostasis is maintained by the combination of absorption from the diet, a modest rate of biosynthesis, and efficient renal reabsorption. Although the renal reabsorption

Inhibitory effect of several compounds on carnitine uptake by isolated mouse enterocytes

Uptake of [ $^3\text{H}$ ]carnitine by isolated mouse enterocytes for 3 min was measured in the presence or absence of each compound. Data represent mean  $\pm$  S.E.M. ( $n = 3$ ). Data obtained in HEK293 cells stably expressing mouse OCTN2 are from our previous report (Ohashi et al., 2001) unless noted otherwise.

| Inhibitors                         | Concentration | Relative Uptake  |                    |
|------------------------------------|---------------|------------------|--------------------|
|                                    |               | Enterocytes      | HEK293/mOCTN2      |
|                                    | $\mu M$       | % of Control     |                    |
| Control                            |               | 100.0 $\pm$ 1.38 | 100                |
| L-Carnitine                        | 5             | 88.6 $\pm$ 1.38* | 83.1*              |
| L-Carnitine                        | 500           | 56.4 $\pm$ 0.99* |                    |
| D-Carnitine                        | 5             | 90.2 $\pm$ 1.60* |                    |
| D-Carnitine                        | 500           | 67.8 $\pm$ 2.39* |                    |
| Acetyl-L-carnitine                 | 5             | 76.8 $\pm$ 0.81* | 67.3 <sup>aa</sup> |
| Acetyl-L-carnitine                 | 500           | 58.2 $\pm$ 2.09* |                    |
| Acetylcholine                      | 500           | 91.7 $\pm$ 1.02* | 79.1*              |
| Acetylcholine                      | 5000          | 67.9 $\pm$ 1.08* | 34.2*              |
| Serotonin                          | 500           | 103.4 $\pm$ 3.14 | 81.4               |
| Dopamine                           | 500           | 92.3 $\pm$ 1.23* | 81.3*              |
| Dopamine                           | 5000          | 80.6 $\pm$ 2.86* | 70.9*              |
| Epinephrine                        | 500           | 101.3 $\pm$ 0.53 | 105.9              |
| Epinephrine                        | 5000          | 108.6 $\pm$ 0.52 | 106.1              |
| Norepinephrine                     | 500           | 94.9 $\pm$ 2.20  | 93.9               |
| Norepinephrine                     | 5000          | 104.6 $\pm$ 0.86 | 70.9*              |
| Histamine                          | 5000          | 97.9 $\pm$ 0.83  | 100.0              |
| Thiamine                           | 500           | 87.4 $\pm$ 0.88* | 77.4*              |
| Thiamine                           | 5000          | 80.5 $\pm$ 1.77* | 65.9*              |
| $\alpha$ -Ketoglutarate            | 500           | 100.3 $\pm$ 0.43 | 96.4               |
| Quinidine                          | 500           | 43.5 $\pm$ 0.10* | 6.1*               |
| Verapamil                          | 500           | 48.7 $\pm$ 1.08* | 1.3*               |
| Guanidine                          | 500           | 97.9 $\pm$ 2.05  | 91.3               |
| N <sup>1</sup> -methylnicotinamide | 500           | 110.6 $\pm$ 1.23 | 99.3               |
| Tetraethylammonium                 | 50            | 61.3 $\pm$ 3.01* | 67.8*              |
| Tetraethylammonium                 | 500           | 54.1 $\pm$ 0.26* | 15.4*              |
| 1-Methyl-4-phenylpyridinium        | 500           | 86.8 $\pm$ 1.79* | 72.9*              |
| Pyrimilamine                       | 500           | 75.2 $\pm$ 1.51* | 36.3*              |
| Diphenhydramine                    | 500           | 102.0 $\pm$ 1.17 | 43.6*              |
| Procainamide                       | 500           | 98.7 $\pm$ 0.77  | 81.3*              |
| Lidocaine                          | 500           | 100.1 $\pm$ 1.98 | 57.1*              |
| para-Aminohippurate                | 500           | 105.9 $\pm$ 1.72 | 100.4              |
| Probenecid                         | 500           | 112.8 $\pm$ 2.54 |                    |

<sup>a</sup> Data from Tamai et al. (2000).

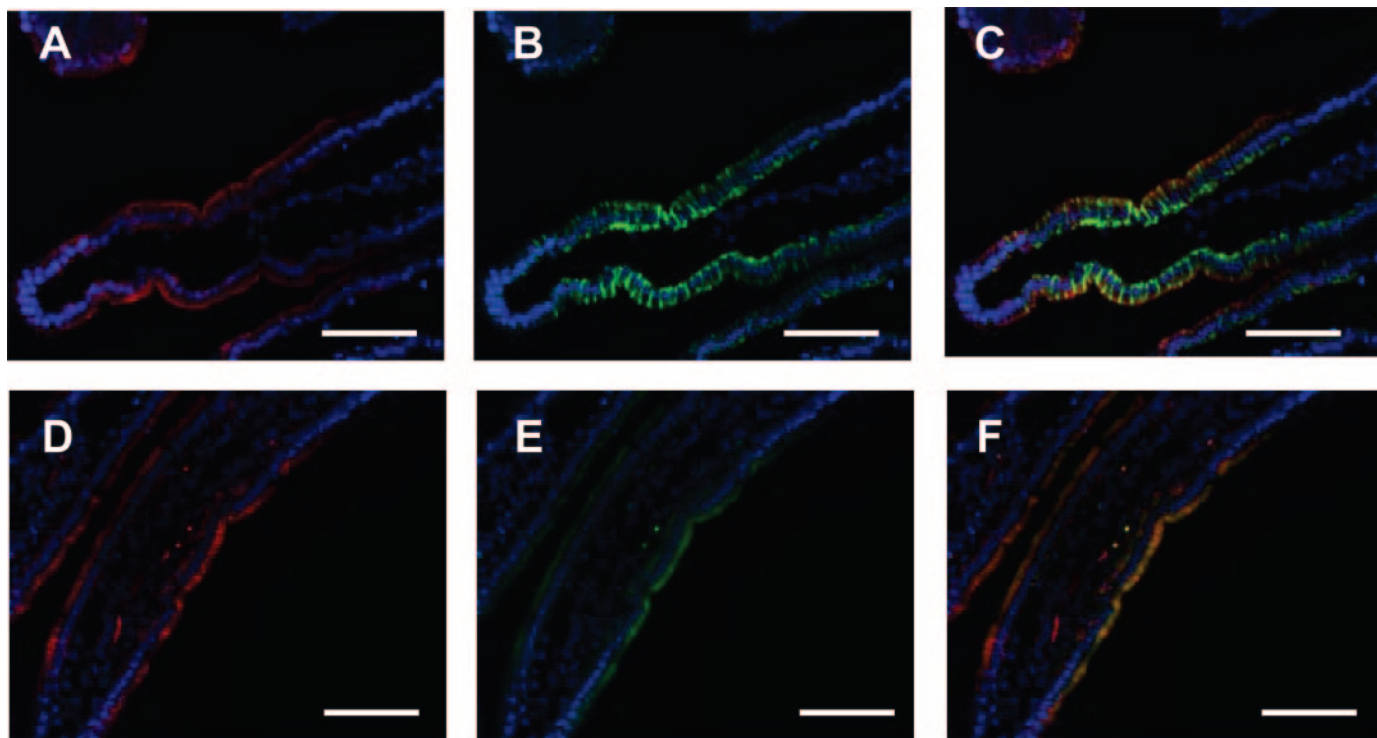
\* Significantly different from control ( $P < 0.05$ ).

of carnitine has been demonstrated to be mainly mediated by OCTN2, the molecular mechanism of small intestinal absorption remains controversial. The present studies demonstrated that uptake of carnitine from apical membranes of small intestinal epithelial cells is mainly governed by OCTN2 in mice, based on the following observations: 1) uptake of carnitine from apical membranes in the small intestine was saturable, decreased by  $\text{Na}^+$ -replacement, and much lower in *jvs* mice with a hereditary deficiency of the *octn2* gene than in wild-type mice (Figs. 1 and 2); 2) the higher uptake of carnitine from the apical side at the middle part of the small intestine is consistent with the higher expression of OCTN2 gene product at this site (Figs. 1, 2, and 7A); 3) uptake of carnitine by isolated enterocytes was also saturable, decreased by  $\text{Na}^+$ -replacement, and much lower in *jvs* mice than in wild-type mice (Figs. 3 and 4); 4) the uptake of carnitine by enterocytes of wild-type mice was mainly governed by a saturable component (Fig. 4), the  $K_m$  value of which is similar to the  $K_m$  value for carnitine uptake in HEK293 cells heterologously expressing mouse OCTN2 (22  $\mu\text{M}$ ; Tamai et al., 2000); 5) the inhibition profile of carnitine uptake by isolated enterocytes of wild-type mouse (Table 1) was similar to that observed in HEK293 cells heterologously expressing mouse OCTN2 (Table 1) (Tamai et al., 2000; Ohashi et al., 2001); and 6) OCTN2 is localized at the brush-border regions of apical membranes in small intestinal absorptive cells (Figs. 5 and 6). The present finding is thus the first demonstration of functional expression and a predominant role in carnitine uptake of OCTN2 at the apical side in mouse small intestine.

Yokogawa et al. (1999) have reported higher bioavailability of carnitine in wild-type mice compared with *jvs* mice. This result is consistent with the present finding that carnitine

uptake from the apical side in the small intestine is predominantly mediated by OCTN2 in mice, if we assume that intestinal absorption of carnitine mainly occurs in the small intestine. It is noteworthy that in an Ussing-type chamber system with small intestinal tissues, uptake of carnitine from the apical side was much higher than that from the basal side in wild-type mice (Fig. 1, A and B), implying that the small intestine plays a role as an absorptive organ for carnitine through preferential transport from the apical to the basal side. This idea is also supported by the localization of OCTN2 in the brush-border of intestinal absorptive cells (Fig. 6A). Such a physiological role is similar to that in the kidney, in which carnitine is reabsorbed mainly by OCTN2 in proximal tubules (Nezu et al., 1999; Yokogawa et al., 1999).

It should be noted that oral bioavailability of carnitine in *octn2* gene-deficient *jvs* mice is still significant ( $\sim 0.34$ ; Yokogawa et al., 1999). This is compatible with the present observation that uptake of [ $^3\text{H}$ ]carnitine from the apical side was observed in small intestine of *jvs* mice (Fig. 1B), and this cannot be accounted for by the extracellular space as assessed in terms of uptake of [ $^{14}\text{C}$ ]inulin (see *Results*). These findings suggest the presence of uptake mechanism(s) other than OCTN2 on the apical membranes of mouse small intestine. Such mechanism(s) probably have low affinity for carnitine, because the uptake found in *jvs* mice was not saturated even in the presence of 20 mM concentration of unlabeled carnitine (Fig. 2, B, D, and F). Among OCTN family members in mice, both OCTN1 and OCTN3 accept carnitine as a substrate. Mouse OCTN3 has higher affinity for carnitine than mouse OCTN2, with the  $K_m$  values of OCTN2 and OCTN3 being 22 and 3  $\mu\text{M}$ , respectively (Tamai et al., 2000). In addition, OCTN3 protein is mainly localized in the basolateral membrane of rat and chicken enterocytes



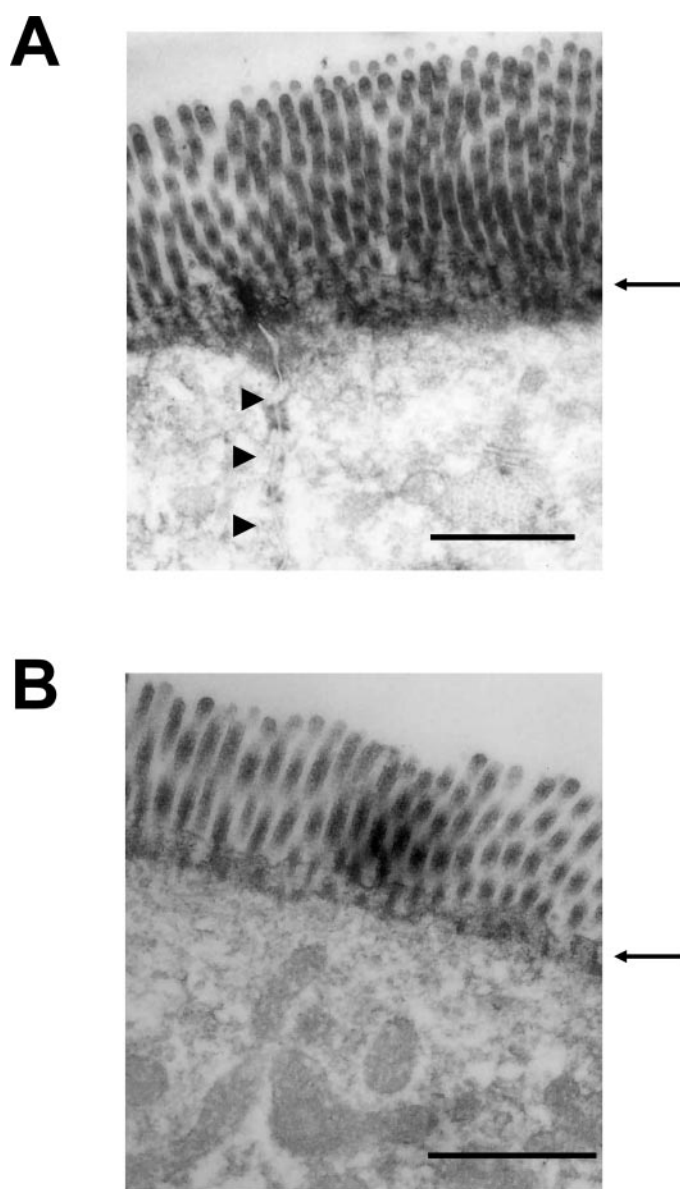
**Fig. 5.** Immunolocalization of OCTN2 in small intestines. Cryosections (10  $\mu\text{m}$ ) of mouse small intestine were double-stained with affinity-purified rabbit antiserum against mOCTN2 (A and D), mouse monoclonal antibody against  $\text{Na}^+$ ,  $\text{K}^+$ , and ATPase (B), and rat antiserum against mouse PDZK1 (E). Overlay images (C and F) show that OCTN2 is colocalized with PDZK1 at the apical membrane of intestinal epithelial cells. Scale bars, 100  $\mu\text{m}$ .



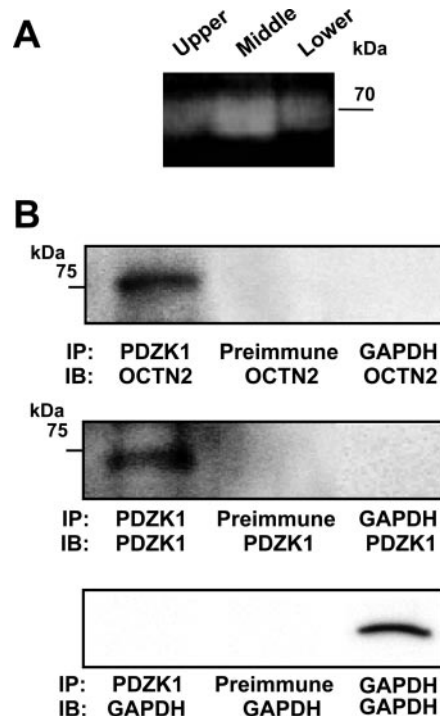
(Duran et al., 2005). Thus, it is unlikely that this transporter is involved in carnitine uptake from apical membranes. On the other hand, human OCTN1 has a lower affinity for carnitine, and the transport activity of human OCTN1 is much lower than that of human OCTN2 (Yabuuchi et al., 1999; Peltekova et al., 2004; Grundemann et al., 2005). We observed faint immunostaining with anti-OCTN1 antibody on apical membranes in mouse small intestine (data not shown). Therefore, OCTN1 is one of the candidates for transporters involved in carnitine uptake across the apical membranes in mouse small intestine. However, there has been no information on specific inhibitors that can distinguish function between OCTN1 and other transporters in mice. Thus, contribution of OCTN1 cannot be proposed in the present inhibition study (Table 1). Another possible candidate could

be the amino acid transporter ATB<sup>0+</sup>, which has low affinity ( $K_m \sim 0.8$  mM) for carnitine and is expressed on the luminal membrane of mouse colonocytes (Nakanishi et al., 2001; Hatanaka et al., 2002), although whether or not it is localized in mouse small intestine remains to be examined.

Immunohistochemical analysis revealed colocalization of OCTN2 with PDZK1 on apical membranes of small intestinal epithelial cells in mice (Fig. 5). Immunoprecipitation and subsequent Western blot analysis have demonstrated interaction of the two proteins in mouse small intestinal mucosa (Fig. 7B). Such colocalization of the two proteins in small intestine is consistent with the observation in proximal tubules in kidney (Kato et al., 2005). PDZK1 interacts with so-called PDZ binding motifs, which are usually located at the carboxyl terminus of various types of membrane proteins (Gisler et al., 2003; Anzai et al., 2004; Kato et al., 2005; Miyazaki et al., 2005). Although the interaction and colocalization of PDZK1 with various types of transporters has been demonstrated in apical membranes of renal proximal tubules (Gisler et al., 2003; Anzai et al., 2004), information on the interaction at apical membranes of the small intestine is still limited. Human PDZK1 directly interacts with the carboxyl terminus of human OCTN2 and has been proposed to stimulate the carnitine transport function of human OCTN2 without altering its cell-surface expression (Kato et al., 2005). Therefore, the present observation of colocalization of the two proteins in brush-border regions of apical membranes in small intestine suggests that functional coupling of PDZK1 and OCTN2 may also occur in mouse small intestine. However, the carboxyl terminus of OCTN2 can interact with



**Fig. 6.** Electron microscopic analysis of localization of OCTN2 (A) and PDZK1 (B) in apical membranes of absorptive cells in small intestine. The immunoreactivity for OCTN2 was mainly localized in microvilli of small intestinal absorptive cells but not in the lateral membranes (arrowheads, A). Similar localization in the microvilli was also observed for PDZK1 (B). In both cases, a part of the immunoreactivity was observed at the base of the microvilli in the apical cytoplasm (arrows). Scale bars, 1  $\mu$ m.



**Fig. 7.** Expression and interaction of OCTN2 with PDZK1 in mouse small intestine. A, lysates of mouse small intestinal mucosa were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-OCTN2 antibody. B, lysates of mouse small intestinal mucosa were immunoprecipitated with anti-PDZK1 antibody, preimmune serum, or anti-GAPDH antibody followed by Western blot analysis using anti-OCTN2 (upper), anti-PDZK1 (middle), or anti-GAPDH (lower) antibodies.

other PDZ domain-containing proteins, including IKEPP/PDZK2 (Kato et al., 2005), which is also expressed on apical membranes of small intestinal epithelial cells (Scott et al., 2002). Our recent observations have indicated the modulation of human OCTN2 expression by human IKEPP/PDZK2 (C. Watanabe, Y. Kato, T. Sugiura, Y. Kubo, T. Wakayama, S. Iseki, and A. Tsuji, submitted for publication). Thus, identification of the whole molecular machinery for carnitine uptake still requires further studies.

Human and mouse OCTN2 accepts various types of organic cations, including quinidine, verapamil, and pyrilamine, as substrates, although the uptake of such cationic compounds observed in a transfectant system was not large compared with that of carnitine (Ohashi et al., 1999, 2001; Wu et al., 1999; Tamai et al., 2000; Grundemann et al., 2005). Therefore, functional expression of OCTN2 on the apical membranes of small intestine suggests that these transporters could be promising targets for improving the gastrointestinal absorption of certain types of therapeutic agents. Involvement of transporters in intestinal absorption of therapeutic agents has recently been recognized, although direct evidence using transporter gene-deficient animals has not yet been obtained. For example, because we proposed saturable uptake mechanism(s) for at least certain types of penicillins and  $\beta$ -lactam antibiotic compounds in the small intestine in the 1970s (Tsuji et al., 1977), many studies have been performed to clarify the involvement of oligopeptide transporters, possibly including PEPT1, in gastrointestinal absorption of various therapeutic agents (Han and Amidon, 2000; Daniel, 2004; Sai and Tsuji, 2004). Recent observations regarding drug-food interactions have suggested functional involvement of certain types of transporters in intestinal absorption of therapeutic agents in vivo (Dresser et al., 2002). Thus, the present observation should prompt further analysis of possible roles of OCTN2 in intestinal absorption of substrates other than carnitine.

Electron microscopic analysis has revealed that OCTN2 and PDZK1 are localized at the base of microvilli, and such a localization is similar to that of the terminal web of actin filaments (Fig. 6, A and B). Although the exact localization of PDZK1 in small intestinal absorptive cells is not fully established, the present observation may be compatible with the previous finding that PDZK1 is colocalized with  $\beta$ -actin and is also present in the intermicrovillar clefts of mouse renal proximal tubules (Gisler et al., 2001). PDZK1 can directly interact with NHERF1, which would be indirectly associated with actin through ezrin (Gisler et al., 2003; Wade et al., 2003). Therefore, a part of OCTN2 might be colocalized with PDZK1 in the cytoplasmic region, and further studies are necessary to identify the pharmacological significance of such heterologous localization of the transporter. In conclusion, the present findings demonstrate that OCTN2 is predominantly responsible for the uptake of carnitine from the apical surface of small intestinal epithelial cells in mice.

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