

PDZK1 Regulates Breast Cancer Resistance Protein in Small Intestine

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ABSTRACT:

Transporter adaptor protein PDZK1 regulates several influx transporters for xenobiotics and nutrients in small intestine, and their expression on the apical membrane is diminished in *pdzk1* gene knockout [*pdzk1*(-/-)] mice. In the present study, we initially attempted to use *pdzk1*(-/-) mice to functionally identify influx transporters responsible for intestinal absorption of cimetidine. Contrary to our expectation, the plasma concentration of cimetidine after oral administration to *pdzk1*(-/-) mice was higher than that in wild-type mice, and the double peaks of plasma concentration found in wild-type mice were not observed in *pdzk1*(-/-) mice. Western blot analysis of intestinal brush-border membranes revealed that expression of breast cancer resistance protein (BCRP) but not of P-glycoprotein is reduced in *pdzk1*(-/-) mice. This result was compatible with the reduction of apical localization of BCRP in *pdzk1*(-/-) mice assessed by immunohistochemical

analysis. Transcellular transport of cimetidine in the basal-to-apical direction in Madin-Darby canine kidney II (MDCKII) cells stably expressing both BCRP and PDZK1 (MDCKII/BCRP/PDZK1) was higher than that in MDCKII cells stably expressing BCRP (MDCKII/BCRP) cells. Moreover, MDCKII/BCRP/PDZK1 cells are more resistant than MDCKII/BCRP cells to the cytotoxicity of the anticancer agent 7-ethyl-10-hydroxycamptothecin (SN-38), which is a substrate of BCRP. These results were consistent with the higher expression of BCRP on apical membranes in MDCKII/BCRP/PDZK1 cells. Pull-down and immunoprecipitation studies revealed a physical interaction between BCRP and PDZK1. Taken together, these findings demonstrate that PDZK1 plays a pivotal role in the apical localization of BCRP. This is the first identification of a regulatory protein that physically interacts with and regulates BCRP in small intestine *in vivo*.

Introduction

Orally administered drugs must permeate across the plasma membranes of intestinal epithelial cells before entering the systemic circulation. Membrane permeation of lipophilic drugs generally occurs by simple diffusion through the lipid bilayer, but absorption of some drugs is hindered by efflux transporters localized on the apical membranes. These include multidrug resistance protein (MDR1/P-glycoprotein/ABCB1) and breast cancer resistance protein (BCRP/ABCG2), both of which are known to act a barrier against intestinal absorption of various therapeutic agents *in vivo* (Greiner et al., 1999; Kuppens et al., 2007; Schwarz et al., 2007; Yamasaki et al., 2008;

Keskitalo et al., 2009). On the other hand, intestinal membrane permeation of some drugs is mediated by influx transporters that belong to the solute carrier (SLC) superfamily. They include oligopeptide transporter (PEPT) and organic anion-transporting polypeptides (OATPs) (Chu et al., 2001; Dresser et al., 2002). However, the influx mechanisms for most orally administered drugs have not yet been identified.

Several studies have revealed that a postsynaptic density 95/disc-large/zona occludens (PDZ) domain-containing protein, PDZK1, interacts with several SLC transporters in small intestine (Hillesheim et al., 2007; Sugiura et al., 2008, 2010). In *pdzk1* gene knockout [*pdzk1*(-/-)] mice, localization of influx transporters for xenobiotics and nutrients on apical membranes is greatly reduced, with a concomitant reduction or delay in intestinal absorption of their substrates (Sugiura et al., 2008, 2010), indicating that PDZK1 plays a key role in the localization and/or intracellular sorting of those transporters *in vivo*. Such PDZK1-interacting transporters include the sodium/proton

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ABBREVIATIONS: MDR, multidrug resistance protein; ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; SLC, solute carrier; PEPT, oligopeptide transporter; OATP, organic anion-transporting polypeptide; PDZ, postsynaptic density 95/disc-large/zona occludens; OCT, organic cation transporter; SN-38, 7-ethyl-10-hydroxycamptothecin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BBMV, brush-border membrane vesicle; RIPA, radioimmunoprecipitation assay; PBS, phosphate-buffered saline; MDCKII, Madin-Darby canine kidney II; MDCKII/BCRP, MDCKII cells stably expressing BCRP; MDCKII/BCRP/PDZK1, MDCKII cells stably expressing both BCRP and PDZK1; BL, basal; AP, apical; GST, glutathione transferase; OAT, organic anion transporter; MRP, multidrug resistance-associated protein; SNP, single nucleotide polymorphism; URAT, uric acid/anion exchanger.

exchanger NHE3/SLC9A3, oligopeptide transporter PEPT1/SLC15A1, carnitine/organic cation transporter OCTN2/SLC22A5, and organic anion-transporting polypeptide OATP1A/SLCO1A, all of which have a so-called PDZ binding motif at their C-terminal domain (Sugiura et al., 2008, 2010; Zachos et al., 2009). PDZK1 has four PDZ domains in its structure, and each PDZ domain can interact with a PDZ-binding motif.

Although *in vivo* evidence for the interaction of PDZK1 with transporters is still quite limited, it has been suggested that PDZK1 may potentially interact with a large number of influx transporters and other membrane proteins, on the basis of *in vitro* findings (Gisler et al., 2003; Anzai et al., 2004; Kato et al., 2004). In consideration of the wide range of influx transporters interacting with PDZK1, it is possible that PDZK1 interacts with so-far unidentified transporters involved in the gastrointestinal membrane permeation of certain drugs. If so, absorption of those drugs should be decreased or delayed in *pdzk1(-/-)* mice compared with wild-type mice. Hence, *pdzk1(-/-)* mice could be a unique tool to functionally clarify the involvement of intestinal influx transporters in drug absorption and might be useful for identifying novel transporters in the small intestine. In the present study, we attempted to use *pdzk1(-/-)* mice to identify the small intestinal influx transporter for cimetidine.

Cimetidine is orally administered as a histamine H₂-receptor antagonist to reduce gastric acid secretion for the treatment of gastric ulcer. It is well absorbed after oral administration, but the intestinal influx mechanism for this compound is still not established. It has been reported that simple diffusion and/or paracellular permeation is involved in gastrointestinal absorption of cimetidine (Zhou et al., 1999; Piyapolrunroj et al., 2000), although cimetidine should be present at least partly in ionized form at the mucosal microclimate pH, based on its reported pK_a value of 6.93 (Avdeef and Berger, 2001). Cimetidine is a class 3 compound according to the Biopharmaceutics Drug Disposition Classification System. Shugarts and Benet (2009) proposed that influx transporters such as OATPs could be involved in intestinal absorption of class 3 compounds, as evidenced by the effect of fruit juices on oral absorption of atenolol and fexofenadine (Dresser et al., 2002; Lilja et al., 2005). Furthermore, several class 3 compounds are substrates for intestinal efflux transporters. In fact, cimetidine is a substrate of MDR1 and BCRP (Lentz et al., 2000; Pavsek et al., 2005). Involvement of MDR1 and unidentified organic cation/H⁺ antiport systems was also suggested in the secretion of cimetidine in small intestine (Piyapolrunroj et al., 1999; Dahan and Amidon, 2009).

In the present study, we initially compared the plasma concentration-time profile of cimetidine after oral administration between wild-type and *pdzk1(-/-)* mice. An unexpected finding was that gastrointestinal absorption of cimetidine in *pdzk1(-/-)* mice was somewhat higher than that in wild-type mice, implying possible regulation of an efflux transport system(s) for cimetidine by PDZK1. Further analyses indicated that PDZK1 plays a pivotal role in the apical localization of BCRP.

Materials and Methods

Materials. Cimetidine and 7-ethyl-10-hydroxycamptothecin (SN-38) were purchased from Wako Pure Chemicals (Osaka, Japan) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. cDNA clones encoding human PDZK1 with an N-terminal myc tag were subcloned into the pBudCE4.1 vector (Invitrogen, Carlsbad, CA). Monoclonal antibodies against human BCRP (BXP-21), mouse BCRP (BXP-53), MDR1 (C219), PDZK1 (42/clamp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β -actin (AC-15) were obtained from Kamiya Biomedical (Seattle, WA), Sanbio (Am Uden, The Netherlands), Dako North America Inc. (Carpinteria, CA), BD (Franklin Lakes, NJ), Millipore Bioscience Research Reagents (Temecula, CA), and Sigma-Aldrich (St. Louis, MO), respectively. Polyclonal antibody against

caveolin-1 was obtained from Sigma-Aldrich. All other reagents were commercial products of reagent grade.

Animals. Male mice were used for experiments at 6 to 9 weeks of age. The *pdzk1(-/-)* mice had been produced previously (Lan and Silver, 2005). *pdzk1(-/-)* and littermates were of a mixed genetic background (C57BL/6J and 129Sv/Ev). They were maintained with free access to food and water. This study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* at the Takara-machi Campus of Kanazawa University.

Pharmacokinetic Studies in Mice. Mice were fasted overnight with free access to water and anesthetized by intraperitoneal injection of pentobarbital. Cimetidine dissolved in saline was intravenously or orally administered at 40 or 100 mg/kg, respectively. At various intervals after the administration, aliquots of blood were collected through the caudal vein. All blood samples were immediately centrifuged to obtain plasma, which was further mixed with 4 volumes of methanol and then centrifuged, and the resultant supernatant was used for quantification.

Western Blot Analysis of Intestinal Brush-Border Membrane Vesicles. Small intestinal BBMVs were prepared from wild-type mice according to a previous report and solubilized in RIPA-Y buffer containing 1% Nonidet P-40, 75 mM NaCl, 50 mM Tris-HCl, pH 7.5, and protease inhibitors (Sugiura et al., 2008). The lysates were then analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting as described previously (Sugiura et al., 2008) using anti-PDZK1 or anti-mouse BCRP antibodies.

Immunohistochemical Analysis. Frozen sections of mouse small intestine were prepared as described previously (Sugiura et al., 2008). After successive pretreatments with 0.3% H₂O₂ in methanol and 5% bovine serum albumin in PBS, the sections were incubated with anti-mouse BCRP antibody and then washed with PBS. The immunoreaction product was visualized by incubating the sections successively with biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA) for 2 h, a VECTASTAIN Elite ABC Standard Kit (Vector Laboratories) for 30 min, and a 3',3'-diaminobenzidine tetrahydrochloride Peroxidase Substrate Kit (ImmPACT; Vector Laboratories).

Transport Studies in MDCKII Cells. MDCKII cells stably expressing BCRP (MDCKII/BCRP) were constructed at GenoMembrane Inc. (Yokohama, Japan). In brief, cDNA encoding human BCRP was subcloned into pcDNA3.1 vector and transfected into MDCKII cells using Lipofectamine 2000. Stably transfected cells were selected by adding G418 (Sigma-Aldrich) to the culture medium to obtain MDCKII/BCRP cells. MDCKII/BCRP cells were further transfected with pBudCE4.1/PDZK1, and stably transfected cells were selected by addition of both G418 and phleomycin (Zeocin; Invitrogen) to obtain MDCKII/BCRP/PDZK1 cells, which were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 1% sodium pyruvate, 100 unit/ml benzylpenicillin, 0.14 mg/ml streptomycin, 1 mg/ml G418, and 0.2 mg/ml Zeocin in a humidified atmosphere of 5% CO₂ in air at 37°C. MDCKII/PDZK1 cells were obtained by transfection of pBudCE4.1/PDZK1 in MDCKII cells.

MDCKII, MDCKII/BCRP, and MDCKII/BCRP/PDZK1 cells were seeded in Transwell polycarbonate inserts (3- μ m pore size, 12-mm diameter; Corning Life Sciences, Edison, NJ) at a density of 3×10^5 cells/well. After 3 days of culture, the cell monolayers were washed twice with transport buffer (Hanks' balanced salt solution, pH 7.2). The transport buffer containing cimetidine was added to the basal (BL) or apical (AP) chamber. At the designated times, a 100- μ l aliquot was sampled from the opposite side and replaced with an equal volume of fresh buffer.

The efflux ratio of drugs across cell monolayers was calculated as the ratio of the apparent permeability coefficients (P_{app}) in the BL-to-AP direction to that in the AP-to-BL direction, where P_{app} was calculated as the slope of the regression line on the transport-time profile of the drug divided by the initial drug concentration in the donor chamber and the cell monolayer surface area (1.1 cm²).

Cytotoxicity Studies in MDCKII Cells. MDCKII, MDCKII/BCRP, and MDCKII/BCRP/PDZK1 cells were seeded in 24-well plates at a density of 1×10^4 cells/well. After a 24-h incubation period, various concentrations of SN-38 (0.01–30 μ M) were added. After incubation for 5 days, the cell growth in each well was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay.

Cell Surface Biotinylation Assay in MDCKII Cells. MDCKII/BCRP and MDCKII/BCRP/PDZK1 cells were washed twice with ice-cold PBS contain-

ing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-calcium/magnesium) and incubated with 1 mg/ml sulfo-NHS-SS-biotin (Thermo Fisher Scientific, Waltham, MA) at 4°C for 2 h. The cells were washed with quenching buffer (100 mM glycine in PBS-calcium/magnesium) and further incubated at 4°C for 30 min to remove unreacted biotin. Then, cells were washed twice with PBS-calcium/magnesium, harvested, and solubilized in RIPA-Y buffer. Streptavidin-agarose beads (Thermo Fisher Scientific) were added to the lysate followed by incubation at 4°C overnight with end-over-end rotation. The beads were washed twice with RIPA-Y buffer and twice with PBS-calcium/magnesium. The biotinylated proteins were eluted from the beads with SDS loading buffer. The biotinylated proteins and whole-cell lysate were subjected to Western blot analysis.

Immunocytochemical Analysis in MDCKII Cells. The cells were grown on cover glass (15 × 15 mm, thickness 0.12–0.17 mm; Matsunami Glass Inc., Osaka, Japan). Cells were fixed with 3% formaldehyde in PBS, permeabilized with methanol for 10 min, incubated with anti-human BCRP antibody for 1 h at room temperature, washed with PBS and further incubated with Alexa Fluor 488 goat anti-mouse IgG conjugate at a dilution of 1:200 in PBS containing 5% skim milk and 3% bovine serum albumin. They were finally sealed onto the slides using Dapi-Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and the fluorescence was detected with a confocal laser scanning microscope (LSM 710; Carl Zeiss Inc., Oberkochen, Germany).

Pull-Down Studies Using Recombinant GST Fusion and His-Tagged Proteins. C-terminal domains of human OATP1A2 and organic anion transporter (OAT) 1 were subcloned into pGEX6P-1 vector. Recombinant proteins for their GST fusions (GST-OATP1A2 and GST-OAT1, respectively) and His₆-tagged PDZK1 were obtained as described previously (Kato et al., 2004). Lysate of MDCKII/BCRP was incubated for 3 h at 4°C with the GST fusion protein and His₆-tagged PDZK1. The mixture was further incubated for 3 h with glutathione-Sepharose 4B and then washed four times with ice-cold PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-human BCRP and anti-PDZK1 antibody.

Immunoprecipitation Analysis in MDCKII Cells. Lysate of MDCKII/BCRP/PDZK1 was incubated for 1.5 h at 4°C with anti-human BCRP antibody or control mouse IgG. Protein A-Sepharose (GE Healthcare, Uppsala, Sweden) was added, and the mixture was incubated for 1 h, followed by centrifugation and washing three times with PBS to obtain immune complexes. The samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-PDZK1 or anti-human BCRP antibody.

High-Performance Liquid Chromatography Analysis. The high-performance liquid chromatography system consisted of a constant flow pump (LC-10Avp; Shimadzu, Kyoto, Japan), a UV detector (SPD-M10Avp; Shimadzu), an automatic sample injector (SIL-10A; Shimadzu), and an integrator (CLASS-VP; Shimadzu). The reverse-phase column (Cosmosil 5C18-AR-II, 4.6 × 150 mm; Nacal Tesque, Kyoto, Japan) was maintained at 40°C in a column oven (CTO-10Avp; Shimadzu). For cimetidine, the mobile phase was a mixture of 10 mM potassium phosphate buffer (pH 8.0) and acetonitrile (88:12), and the UV detector was set at 228 nm. The flow rate was 1.0 ml/min. Famotidine was used as an internal standard.

Statistical Analysis. Statistical analyses were performed by means of Student's *t* test or analysis of variance with a Tukey post hoc comparison test for single and multiple comparisons, respectively. Differences were considered statistically significant at *p* < 0.05.

Results

Intestinal Absorption of Cimetidine Was Higher in *pdzk1*(*-/-*) Mice.

After oral administration of cimetidine, the plasma concentration-time profile exhibited double peaks in wild-type mice (Fig. 1A). This result is consistent with a previous report (Oberle and Amidon 1987). On the other hand, the plasma concentration in *pdzk1*(*-/-*) mice was monomodal and significantly higher than that in wild-type mice (Fig. 1A). Plasma concentration-time profiles after intravenous administration were similar in wild-type and *pdzk1*(*-/-*) mice (Fig. 1B), showing comparable systemic elimination. The mean value for terminal half-life in wild-type mice was 62.4 and 16.3 min after oral and intravenous administration, respectively, implying that the absorption phase was still observed during 120 min after oral administration (Fig. 1). Thus, gastrointestinal absorption of cimetidine was higher in *pdzk1*(*-/-*) mice than in wild-type mice.

Reduced Expression of BCRP in Small Intestine of *pdzk1*(*-/-*) Mice.

The higher gastrointestinal absorption of cimetidine in *pdzk1*(*-/-*) mice may imply regulation of efflux transporter(s) by PDZK1 in small intestine, because cimetidine is a substrate of efflux transporters BCRP and MDR1 (Lentz et al., 2000; Pavek et al., 2005). To examine the change in expression of these efflux transporters in *pdzk1*(*-/-*) mice, we prepared BBMVs from small intestine and performed Western blot analysis. An immunoreactive band of BCRP was detected in BBMVs prepared from wild-type mice, whereas BCRP was minimally found in BBMVs from *pdzk1*(*-/-*) mice (Fig. 2A). In contrast, the expression levels of MDR1 and β -actin were not remarkably different between the two strains (Fig. 2A).

We further performed immunohistochemical analysis to investigate the change in expression and localization of BCRP in the small intestine (Fig. 2B). BCRP was localized on the apical brush-border membrane in wild-type mice (Fig. 2B, left), but the staining in *pdzk1*(*-/-*) mice was much weaker and broader (Fig. 2B, right) compared with that in wild-type mice.

Effect of PDZK1 on Cell Surface Expression and Localization of BCRP in MDCKII Cells.

To confirm the effect of PDZK1 on BCRP, MDCKII/BCRP/PDZK1, and MDCKII/BCRP cells were constructed to compare the membrane expression of BCRP. A cell surface biotinylation assay was first performed. The efficacy of the biotinylation was validated by assessing the expression levels of caveolin-1 and GAPDH, which are marker proteins of apical membrane and

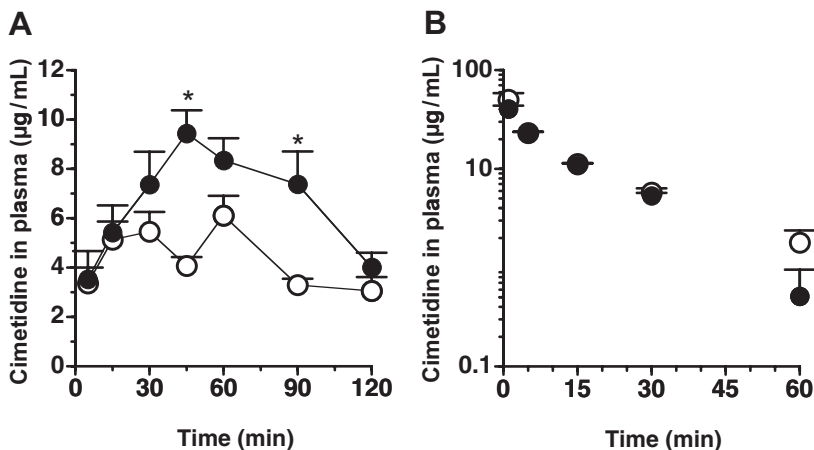


FIG. 1. Plasma concentration-time course of cimetidine after oral (A) and intravenous (B) administration. Cimetidine was orally (100 mg/kg, A) or intravenously (40 mg/kg, B) administered to wild-type (○) and *pdzk1*(*-/-*) (●) mice. Serial blood samples were collected at designated times, and the concentration of cimetidine in plasma was determined. Each value represents the mean ± S.E.M. (*n* = 3–5). *, significant difference from wild-type mice (*p* < 0.05).

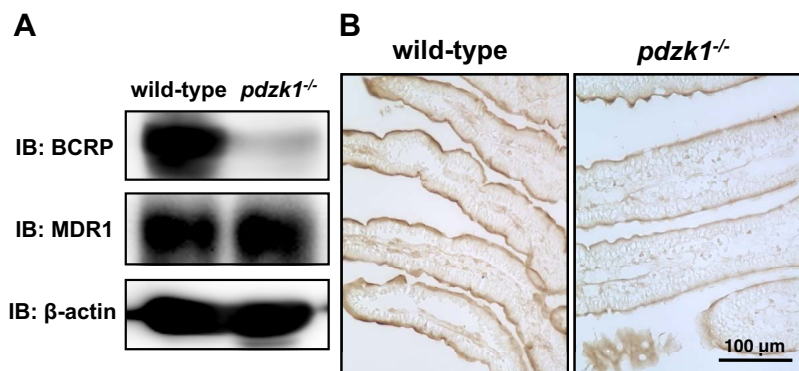


FIG. 2. Reduced expression of BCRP in small intestine of *pdzk1*^{-/-} mice. A, lysate of intestinal BBMVs prepared from wild-type and *pdzk1*^{-/-} mice was subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis using anti-mouse BCRP, MDR1, and β-actin antibodies. B, cryosections of small intestine from wild-type and *pdzk1*^{-/-} mice were immunostained with anti-mouse BCRP antibody. Note that a typical staining pattern of BCRP was observed in apical membrane in wild-type mice, but the intensity of staining was reduced and the staining was broadened in *pdzk1*^{-/-} mice. Original magnification, 200×. IB, immunoblot.

cytoplasm, respectively. Caveolin-1 but not GAPDH was detected in the membrane surface fraction from both cell lines (Fig. 3A, middle and bottom), suggesting minimal contamination of cytoplasmic protein in the biotinylated fraction. Cell surface expression of BCRP on the apical membranes of MDCKII/BCRP/PDZK1 cells was higher than that in MDCKII/BCRP cells (Fig. 3A, top). Densitometric analysis revealed a 3-fold difference in the BCRP expression between the two cell lines. Localization of BCRP was further examined by means of immunocytochemical analysis. BCRP was mainly localized in apical membranes of MDCKII/BCRP/PDZK1 cells (Fig. 3B, right). BCRP in MDCKII/BCRP cells was localized in apical membranes but was also detected in intracellular spaces (Fig. 3B, left). Thus, PDZK1 increases expression of BCRP on apical membranes in MDCKII cells.

Functional Analysis of BCRP in MDCKII/BCRP/PDZK1 Cells. To investigate the functional change in BCRP in the presence of PDZK1, transcellular transport of cimetidine was examined in both MDCKII/BCRP/PDZK1 and MDCKII/BCRP cells. The P_{app} of cimetidine in the BL-to-AP direction across MDCKII/BCRP cells was higher than that across MDCKII cells, indicating the operation of BCRP-mediated transport of cimetidine (Fig. 4A). The P_{app} in the BL-to-AP direction across MDCKII/BCRP/PDZK1 cells was higher than that in MDCKII/BCRP cells, whereas that in the AP-to-BL direction was similar in the two cell lines (Fig. 4A). The transcellular transport in both directions was almost similar between MDCKII/PDZK1 and MDCKII cells (Fig. 4). The values of the efflux ratio, defined as $P_{app, BL-to-AP}/P_{app, AP-to-BL}$, were 1.03 ± 0.11 , 0.78 ± 0.18 , 1.97 ± 0.17 , and 2.67 ± 0.43 (mean \pm S.E.M. of 6–10 independent monolayers) in MDCKII, MDCKII/PDZK1, MDCKII/BCRP, and MDCKII/BCRP/PDZK1 cells, respectively (Fig. 4). Thus, the transcellular transport of cimetidine was stimulated in the presence of PDZK1.

We next examined the effect of PDZK1 on BCRP-mediated drug resistance to the anticancer agent SN-38 (Fig. 5), because BCRP is one of the key molecules for multidrug resistance. MDCKII/BCRP and MDCKII/BCRP/PDZK1 but not MDCKII/PDZK1 cells were resistant to SN-38, compared with MDCKII cells. The SN-38 concentration causing 50% inhibition of cell growth (EC_{50}) in MDCKII/BCRP/PDZK1 cells (1.77×10^3 nM) was 2.5-fold higher than that in MDCKII/BCRP cells (7.04×10^2 nM) (Fig. 5).

Physical Interaction between BCRP and PDZK1. To investigate the physical interaction between the two proteins, pull-down studies using recombinant GST-OATP1A2 and His₆-tagged PDZK1 were performed. We have already reported that PDZK1 interacts with OATP1A2 but not with OAT1 (Kato et al., 2004). Therefore, GST-OATP1A2 but not GST-OAT1 is expected to bind to His₆-tagged PDZK1. The complex of GST-OATP1A2 and His₆-tagged PDZK1 may then bind to BCRP if there is a physical interaction between PDZK1 and BCRP, affording a ternary complex that can be precipi-

tated with glutathione-Sepharose beads (see *Materials and Methods*). Pull-down using both GST-OATP1A2 and His₆-tagged PDZK1 precipitated a single band corresponding to His₆-tagged PDZK1, but use of both GST-OAT1 and His₆-tagged PDZK1 could not precipitate it (Fig. 6A, middle), supporting the specific interaction of PDZK1 and OATP1A2. Furthermore, pull-down using both GST-OATP1A2 and His₆-tagged PDZK1 resulted in a single band of 75 kDa, which was immunoreactive with anti-human BCRP antibody (Fig. 6A, top). This band was not detected when both His₆-tagged PDZK1 and GST-OAT1 were used (Fig. 6A, top). Pull-down using GST-OATP1A2 alone did not exhibit any immunoreactive band corresponding to BCRP (Fig. 6A, top), suggesting that the C terminus of OATP1A2 cannot directly interact with BCRP. Pull-down using His₆-tagged PDZK1 alone did not exhibit the BCRP band (Fig. 6A, top) probably because the complex of His₆-tagged PDZK1 and BCRP cannot be pulled down due to the absence of a GST fusion protein.

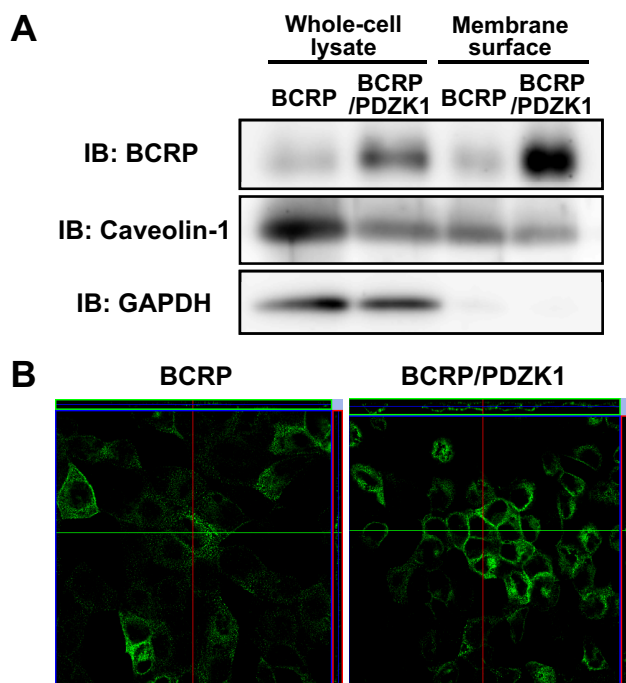


FIG. 3. Effect of PDZK1 on cell surface expression and localization of BCRP in MDCKII cells. A, a cell surface biotinylation assay was performed in MDCKII/BCRP/PDZK1 and MDCKII/BCRP cells. The cell surface proteins and whole-cell lysate were analyzed by Western blot analysis using anti-human BCRP (top), caveolin-1 (middle), and GAPDH (bottom) antibodies. B, MDCKII/BCRP/PDZK1 and MDCKII/BCRP cells were fixed and immunostained with anti-BCRP antibody. Note that the staining of BCRP was localized on the apical membrane in MDCKII/BCRP/PDZK1 cells, whereas the staining was also observed in the intracellular compartment in MDCKII/BCRP cells. IB, immunoblot.

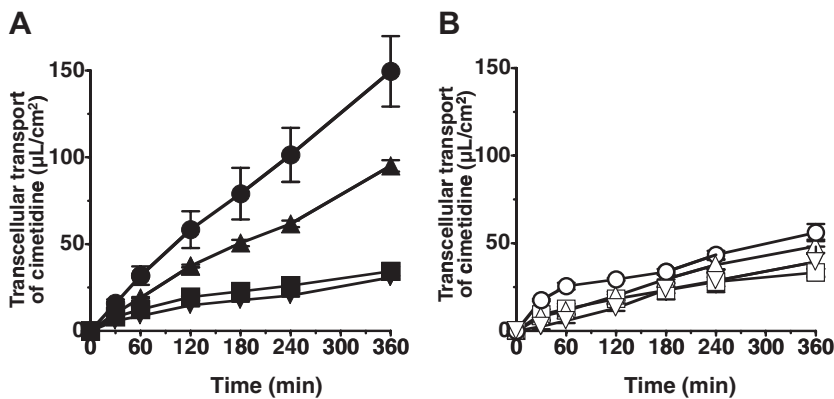


Fig. 4. Effect of PDZK1 on transcellular transport of cimetidine across MDCKII cells stably expressing BCRP. Transport of cimetidine ($100 \mu\text{M}$) in the BL-to-AP (A) and AP-to-BL (B) directions was measured across MDCKII/BCRP/PDZK1 (● and ○), MDCKII/BCRP (▲ and △), MDCKII/PDZK1 (▼ and ▽), and MDCKII (■ and □) cells. Each value represents the means \pm S.E.M. ($n = 4-10$).

We next performed immunoprecipitation of lysates of MDCKII/BCRP/PDZK1 cells using anti-BCRP and anti-PDZK1 antibodies (Fig. 6B). PDZK1 was detected in immunoprecipitates with anti-BCRP antibody but not with control IgG (Fig. 6B, top). The immunoprecipitate obtained with anti-BCRP antibody was also reactive with anti-BCRP antibody, whereas that obtained by control IgG showed no response (Fig. 6B, bottom). These results suggest that BCRP interacts with PDZK1.

Discussion

BCRP is expressed at brush-border membranes in intestinal epithelial cells and transports various xenobiotics from cytoplasm into lumen. Such intestinal drug efflux mediated by BCRP may hinder gastrointestinal absorption of certain therapeutic agents, including sulfasalazine and topotecan (Kuppens et al., 2007; Yamasaki et al., 2008), as suggested by analysis of *ABCG2/BCRP* gene polymorphisms in combination with the use of BCRP-inhibitory drugs. Nevertheless, there have been few reports regarding post-transcriptional regulatory mechanisms involving physical interaction with BCRP in the small intestine. Our present results indicate that PDZK1 regulates BCRP by increasing its expression level on the apical cell surface, because of the following. 1) Expression of BCRP in BBMVs of *pdzkl(-/-)* mice was much lower than that of wild-type mice (Fig. 2A). 2) Localization of BCRP on small intestinal apical membranes in *pdzkl(-/-)* mice was also much lower (Fig. 2B). 3) These two findings in vivo are consistent with the outcome when PDZK1 was

coexpressed with BCRP in MDCKII cells in vitro; i.e., expression of BCRP on apical membrane in MDCKII/BCRP/PDZK1 cells was higher than that in MDCKII/BCRP cells (Fig. 3A), and localization of BCRP on apical membranes was enhanced in MDCKII/BCRP/PDZK1 cells (Fig. 3B). 4) The higher expression of BCRP in MDCKII/BCRP/PDZK1 cells is functionally supported by the higher transcellular transport of cimetidine (Fig. 4) and greater tolerance to cytotoxicity provoked by SN-38 (Fig. 5). 5) PDZK1 physically interacts with BCRP (Fig. 6). All these results are consistent with the regulation of BCRP by PDZK1. It is noteworthy that expression of another intestinal efflux transporter, MDR1, was not affected by gene depletion of *pdzkl* (Fig. 2A), suggesting that the regulation by PDZK1 is specific for BCRP. PDZK1 is reported to directly interact with multidrug resistance-associated protein (MRP) 2, which is expressed in small intestine and has at least partially overlapped substrate specificity with BCRP (Kocher et al., 1999), although there has been no evidence that small intestinal MRP2 is regulated by PDZK1. Post-transcriptional regulation of BCRP has recently been reported in various types of cells (Ikebuchi et al., 2010; Nagai et al., 2011; Sugiyama et al., 2011). Nevertheless, the present findings are the first evidence that PDZK1 is a functional regulator for BCRP in vivo in small intestine.

The plasma concentration profile of cimetidine after oral administration in *pdzkl(-/-)* mice was higher than that in wild-type mice (Fig. 1A). This may reflect the difference in the first-pass effect of cimetidine, because the plasma concentration after intravenous administration was similar in the two strains (Fig. 1B). If we consider that cimetidine is a substrate of both MDR1 and BCRP (Lentz et al., 2000; Pavek et al., 2005) and that BCRP but not MDR1 is down-regulated in *pdzkl(-/-)* mice (Fig. 2), there is a possibility that the higher plasma concentration of cimetidine (Fig. 1A) can be accounted for by the regulation of BCRP by PDZK1 and by a defect in expression of BCRP in the absence of PDZK1 in vivo. As previously reported by Oberle and Amidon (1987), the plasma concentration-time profile of cimetidine after oral administration exhibited a double peak in wild-type mice (Fig. 1A). Other substrates of intestinal efflux transporters, such as cyclosporine and vinblastine, have been reported to show a similar double peak of plasma concentration after oral administration (Reymond et al., 1988; Ogihara et al., 2006), and the double peak disappeared in the presence of transporter inhibitors or after gene knockout of small intestinal efflux transporters (Ogihara et al., 2006). In the present study, the double peak of cimetidine also disappeared in *pdzkl(-/-)* mice, and this finding is consistent with dysfunction of the efflux transporter(s) in *pdzkl(-/-)* mice, leading to a higher plasma concentration of cimetidine than that in wild-type mice (Fig. 1A). However, the possible association between PDZK1 and cimetidine transport should be further examined, because other

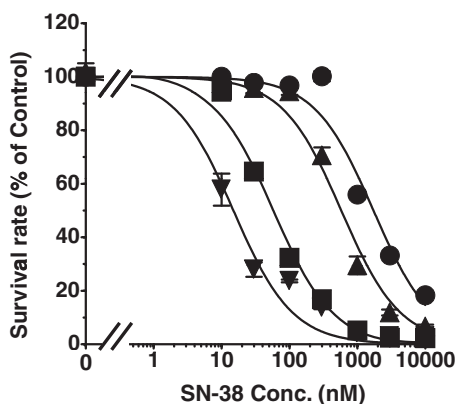


Fig. 5. Effect of PDZK1 on BCRP-mediated drug resistance to SN-38. MDCKII/BCRP/PDZK1 (●), MDCKII/BCRP (▲), MDCKII/PDZK1 (▼), and MDCKII (■) cells were incubated with various concentrations of SN-38 ($0.01-30 \mu\text{M}$) for 5 days, followed by determination of cellular growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Each value represents the mean \pm S.E.M. ($n = 3-9$).

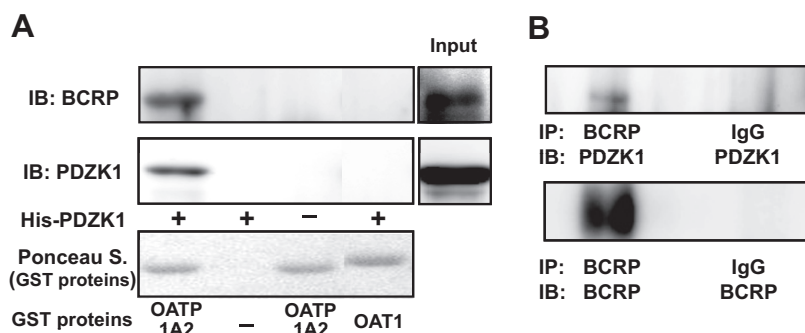


FIG. 6. Interaction of BCRP with PDZK1. A, lysates of MDCKII/BCRP cells were subjected to pull-down analysis with a mixture of His₆-tagged PDZK1 and GST-OATP1A2. GST-OAT1 was also used as a control. The precipitated (interacted) materials were then analyzed by Western blotting using anti-human BCRP and anti-PDZK1 antibodies. The amount of GST fusion protein added to the reaction mixture was checked with Ponceau S. B, lysates of MDCKII/BCRP/PDZK1 cells were immunoprecipitated with anti-human BCRP antibody (left) or control IgG (right), followed by Western blot analysis using anti-PDZK1 (top) and anti-human BCRP (bottom) antibodies. IB, immunoblot; IP, immunoprecipitate.

gastrointestinal events, including a variable gastric emptying rate (Oberle and Amidon, 1987) may also affect the plasma concentration profile after oral administration.

In consideration of the regulation of several types of influx transporters for xenobiotics and nutrients by PDZK1 in small intestine (Sugiura et al., 2008, 2010), the present findings support the novel idea that PDZK1 regulates both influx and efflux transporters. This idea might be consistent with the recent observation of genetic polymorphism in the *PDZK1* gene in humans. It has been reported that single nucleotide polymorphisms (SNPs) in the *PDZK1* gene influence the serum level of uric acid (Kolz et al., 2009; van der Harst et al., 2010). Such an effect of *PDZK1* SNPs on serum uric acid could be explained in terms of regulation by PDZK1 of both influx and efflux transporters for uric acid, e.g., uric acid/anion exchanger URAT1/SLC22A12 and BCRP. URAT1 is expressed on the apical membranes of renal proximal epithelial cells and is involved in renal reabsorption of uric acid. A defect in the *URAT1* gene is associated with idiopathic renal hypouricemia (Enomoto et al., 2002; Ichida et al., 2004). Anzai et al. (2004) already demonstrated direct interaction of this transporter with PDZK1 and regulation by PDZK1. On the other hand, BCRP is a high-capacity secretory transporter for uric acid, and the *ABCG2/BCRP* gene is associated with gout (Matsuo et al., 2009). The genotype combination of two dysfunctional variants Q126X and Q141K results in increased serum uric acid concentration and increased risk of gout (Matsuo et al., 2009). Regulation by PDZK1 of BCRP was demonstrated in the present study. Thus, it is likely that PDZK1 could affect both influx and efflux transports of uric acid, thereby leading to apparently inconsistent effects of *PDZK1* SNPs on serum urate level in humans. A similar hypothesis may also be applicable to the observations in *pdzki(-/-)* mice. Although expression of OCTN2 and PEPT1 on apical membranes of small intestine was almost completely lost in *pdzki(-/-)* mice, the gastrointestinal absorption of their substrates, carnitine and cephalixin, was not completely reduced (Sugiura et al., 2008). One possible explanation is the regulation of unknown efflux transporters for these compounds by PDZK1.

BCRP is highly expressed in certain cancer cells and is thought to be one of the principal factors involved in multidrug resistance. The present study revealed that resistance to cytotoxicity of SN-38 is higher in the presence of PDZK1 (Fig. 5). In consideration of the marked gene expression of PDZK1 in breast and ovarian cancers (Ghosh et al., 2000; Walker et al., 2007), the interaction between BCRP and PDZK1 could be a target for at least partially overcoming BCRP-mediated drug resistance in certain cancer cells.

Transcellular transport of cimetidine in MDCKII/BCRP cells was higher than that in MDCKII cells (Fig. 4). In addition, MDCKII/BCRP cells were more resistant to cytotoxicity of SN-38, compared with MDCKII cells (Fig. 5). These results suggest that BCRP is functionally expressed in MDCKII/BCRP cells even when no PDZK1

is exogenously transfected. In our preliminary study, Western blot analysis using anti-PDZK1 antibody detected a single band at 70 kDa in the lysate of MDCKII/BCRP cells (data not shown), implying that endogenous PDZK1 may regulate the expression of BCRP in this cell line. However, it is also possible that other unidentified adaptor protein(s) may be involved in the expression of BCRP in MDCKII cells.

Interaction of PDZK1 with various transporters occurs at their extreme C-terminal amino acid sequence, which is a class I PDZ binding motif ([S/T]-X-[Φ]; Φ and X mean hydrophobic and any amino acids, respectively). On the other hand, the C-terminal sequence of BCRP, which is K-Y-S in both humans and mice, does not match this motif. The PDZK1-interacting sequence in BCRP has not yet been examined in detail, but it is possible that the interaction between PDZK1 and BCRP is different from those of other apical membrane transporters, such as OCTN2, URAT1, and OATP1A, which interact with PDZK1 via a C-terminal class I PDZ binding motif. BCRP has a large intracellular loop in its N-terminal half-domain (Wang et al., 2008). In addition, certain PDZ domains can bind to an internal PDZ binding motif, which forms a β-hairpin structure (Hillier et al., 1999). Therefore, further studies are necessary to identify the sequence in BCRP that is involved in the interaction with PDZK1. In the present study, we used the complex of the GST fusion protein with the C terminus of OATP1A2, which can interact with PDZK1 (Kato et al., 2004), and His-tagged PDZK1 as bait in the pull-down studies (Fig. 6A) because no information was available on the structural requirement in BCRP for the PDZK1 interaction. Nevertheless, the results of this experiment may support the formation of a tertiary macromolecular complex involving C-terminal OATP1A2, PDZK1, and BCRP (Fig. 6A). Li et al. (2007) have demonstrated spatiotemporal coupling of MRP4 and cystic fibrosis transmembrane conductance regulator via the PDZ adaptor, which could be relevant to cAMP-regulated chloride channel function. Further studies are required to understand the physiological meaning of such a transporter complex.

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Authorship Contributions

Participated in research design: Shimizu, Sugiura, and Kato.
 Conducted experiments: Shimizu, Sugiura, Kijima, and Kato.
 Contributed new reagents or analytic tools: Wakayama, Iseki, and Silver.
 Performed data analysis: Shimizu, Sugiura, Wakayama, Kijima, Nakamichi, Iseki, and Kato.
 Wrote or contributed to the writing of the manuscript: Shimizu, Sugiura, and Kato.

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