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Molecular mechanisms involved in transporter regulation

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ABSTRACT

Drug transporters have been suggested to be involved in various aspects of pharmacokinetics~~, including absorption, distribution and excretion~~. Identification and characterization of drug transporters have given us a scientific basis for understanding drug disposition, as well as the molecular mechanisms of drug interaction and inter-individual/inter-species differences. On the other hand, regulatory mechanisms of drug transporters are still poorly understood, and information is limited to induction and down-regulation of drug transporters by various microsomal enzyme inducers. Little is known about the molecular machinery that directly interacts with the drug transporters. As a first step to clarify such molecular mechanisms, recent studies have identified PDZ (PSD-95/Discs-large/ZO-1) domain-containing proteins that directly interact with the so-called PDZ binding motif located at the C-terminus of drug transporters. Some of the PDZ proteins have been suggested to regulate transporters via at least two pathways, i.e., stabilization at the cell-surface and direct modulation of transporter function. Therefore, it is possible that membrane transport of therapeutic agents is not only governed by the drug transporters themselves, but also indirectly by PDZ proteins. The PDZ proteins are classified as a family, the members of which are thought to have distinct, but also redundant physiological roles. The purpose of this review article is to summarize the available knowledge on protein interactions and functional modulation of drug transporters.

1. Overview

1-1) Introduction

Recent progress in molecular biology has led to the identification of various types of drug transporters which accept therapeutic agents as substrates. Drug transporters are generally classified into the SLC (solute carrier) and ABC (ATP-binding cassette) superfamilies. These drug transporters are believed to be directly involved in ~~various aspects of drug disposition, such as~~ absorption, distribution and excretion of drugs (Sai and Tsuji 2004; Shitara *et al*., 2004; Marzolini *et al*., 2004; Hagenbuch and Meier 2003; Jonker and Schinkel 2004). However, little is yet known about how these transporters are organized in the plasma membranes, and how their expression and function are regulated. Protein-protein interaction is involved in many biological events, including signal transduction, intracellular trafficking, scaffold formation for protein networks, cellular polarization and protein turnover. In this review article, therefore, we summarize recent findings on direct interaction of drug transporters with so-called adaptor proteins that contain PDZ (PSD-95/Discs-large/ZO-1) domains in their structures (Gisler *et al*., 2003; Anzai *et al*., 2004; Kato *et al*., 2004, 2005) and discuss the physiological roles of these PDZ domain containing proteins, including their expression, localization and effect on membrane transporters. Finally, we review the available information on peroxisome proliferator-activated receptor (PPAR)-α as a transcriptional

factor that affects gene expression of both drug transporters and PDZ proteins.

1-2) Significance of identifying protein-transporter interactions

An understanding of protein-protein interactions involving drug transporters would have various advantages. Firstly, it would help to elucidate the molecular machinery that ensures vectorial transport of nutrients and xenobiotics. Epithelial cells, especially in kidney and small intestine, play fundamental roles in the overall absorption of neutrients, and in the secretion of xenobiotics and metabolites (Fig. 1). It is well established that transporters play fundamental roles in such vectorial transport. In renal proximal tubules of rodents, for example, both organic cation transporter (OCT) 1 and OCT2 are localized on basolateral membranes and are involved in uptake of organic cations from circulating blood, whereas organic cation/carnitine transporter (OCTN) 1 and OCTN2 are localized on apical membranes and are proposed to be involved in renal secretion of those compounds into the urine (Jonker and Schinkel 2004; Wright 2005; Ohashi *et al*., 2001; Tamai *et al*., 2004) (Fig. 1). These OCT/OCTN family members recognize various types of organic cations as substrates (Jonker and Schinkel 2004; Wright 2005; Tamai *et al*., 2000) (Fig. 1). OCTN2 and OCTN1 are also involved in reabsorption of carnitine from the tubular duct space (Nezu *et al*., 1999; Tamai *et al*., 1998, 2000)

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(Fig. 1). To obtain such vectorial transport of xenobiotics and nutrients (Fig. 1), OCT/OCTN family members should be appropriately targeted to either basolateral or apical plasma membranes (Fig. 1). ~~However, the molecular mechanisms of intracellular trafficking of the transporters are poorly understood.~~ Protein-protein interaction research may ~~thus~~ throw light on intrinsic mechanisms governing drug absorption and secretion by clarifying intracellular trafficking mechanisms of the transporters.

Certain types of therapeutic agents can also be recognized by the nutrient transporters in the small intestine. For example, β-lactam antibiotics, ACE inhibitors and bestatin are recognized by oligopeptide transporter (PEPT) 1 which is expressed on apical membranes of small intestinal absorptive epithelial cells and thought to be involved in absorption of peptides and therapeutic agents. ~~and renal tubular epithelial cells~~ (Tsuji and Tamai, 1996; Daniel 2004; Nielsen and Brodin 2003; Han and Amidon 2000). Monocarboxylate transporter (MCT) 1 expressed on apical membranes in the small intestine could also be involved in intestinal absorption of anionic compounds (Tsuji and Tamai 1996). Organic anion transporting polypeptide (OATP)-B, which recognizes estrone sulfate as an endogenous substrate, is also expressed on the apical membrane in human small intestine and transports various types of hydrophobic organic anions (Kobayashi *et al*., 2003; Nozawa *et al*., 2004). On the other hand, P-glycoprotein and multidrug resistance associated protein (MRP) 2 are

involved in, the secretion of various types of therapeutic agents in the small intestine (Benet *et al.*, 2004; Kunta and Sinko 2004; Sai and Tsuji 2004). ~~In the liver and kidney, numerous studies have confirmed functional expression of OATP, organic anion transporter (OAT) and OCT/OCTN family members on basolateral and apical surfaces of hepatocytes and renal tubular epithelial cells, where they are involved in vectorial transport of numerous therapeutic agents (Sai and Tsuji 2004; Shitara *et al.*, 2004; Marzolini *et al.*, 2004; Hagenbuch and Meier 2003; Jonker and Schinkel 2004).~~ Protein-protein interaction research may thus uncover intrinsic mechanisms governing drug absorption and secretion ~~in these organs~~, e.g., trafficking and retention of the transporters themselves.

Secondly, adaptor proteins which can bind to drug transporters may affect the cell-surface expression, function and/or intracellular trafficking of the transporters, thereby influencing drug disposition. Pioneering studies have indicated that PDZ (PSD-95/Discs-large/ZO-1) domain-containing proteins such as PDZK1 and Na^+/H^+ exchanger ~~(NHE)~~ regulatory factor (NHERF1, SLC9A3R1) interact with and regulate the function of cystic fibrosis transmembrane conductance regulator protein (CFTR)(Wang *et al.*, 2000; Raghuram *et al.*, 2001). It has also been clarified that accessory proteins such as 4F2hc and CD147 are required for functional expression of neutral amino acid transporter and monocarboxylate transporter (MCT) 1, respectively (Enerson and Drewes

2003; Halestrap and Meredith 2004; Palacin and Kanai 2004). Since the drug transporters are transmembrane proteins whose cell-surface expression should be subject to homeostatic regulation, such regulatory factors may indirectly influence the membrane transport of substrates through interaction with the transporters.

Finally, identification of protein interactions could provide a structural basis for the interactions in networks of transporters and other proteins. In general, active transport requires a driving force, by which a substrate can be concentratively transported against an electrochemical gradient. For example, uptake of substrates by PEPT1 utilizes an inwardly directed H^+ gradient, which is produced by Na^+/H^+ exchanger (NHE). Uptake of substrates by organic anion transporter (OAT) 1 utilizes an outwardly directed gradient of dicarboxylate which is produced by the Na^+ dicarboxylate transporter (NaDC). Therefore, to obtain efficient transporting activity, the appropriate driving force should be produced in close proximity to the drug transporters to avoid diffusive loss of the ion gradient. Indeed, uptake of dipeptides ~~by the H^+/oligopeptide transporter~~ on the apical membrane in Caco-2 cells has been suggested to be indirectly controlled by NHEs, which supply the H^+ gradient for this peptide uptake mechanism(s) (Thwaites *et al.*, 2002). Hence, this oligopeptide transport system exhibits apparent Na^+-dependence in its substrate uptake, and NHE inhibitors hinder uptake of glycylsarcosine (GlySar) in Caco-2 cells

(Thwaites *et al*., 2002; Anderson *et al*., 2003). Functional modulation by NHE can also occur intracellularly, since NHE can export H^+, which is taken up by oligopeptide transporters. Kennedy *et al*. (2002) suggested that optimal absorptive transport of GlySar depends on the NHE activity, and the reliance of an oligopeptide transporter upon a Na^+-dependent intracellular pH regulatory mechanism such as NHE may partly explain the apparent Na^+-dependence of high-affinity dipeptide uptake observed in PEPT2-expressing astrocytes (Kennedy *et al*. 2002). Transporter-adaptor interactions may provide the structural basis of functional coupling of different transporters, as discussed later.

2. PDZ binding motif in drug transporters as a signal for protein interaction

2-1) PDZ interaction motif localized on C-terminus of various drug transporters

PDZ domains are generally composed of 80-90 amino acids, and are defined as domains commonly found in postsynaptic density PSD-95/SAP90, the *Drosophila* septate junction protein disc-large, and the tight junction protein ZO-1 (Hung *et al*., 2002; Biber *et al*., 2004; Brone and Eggermont 2005). PDZ domains are thought to play important roles in targeting of proteins to specific cell membranes, assembling proteins into signaling complexes for efficient transduction, and regulating the function

of transmembrane proteins (Hung *et al*., 2002; Biber *et al*., 2004; Brone and Eggermont 2005). PDZ domains bind to the PDZ binding motif usually located at the C-terminus of interacting proteins. The binding specificity of PDZ domains is critically determined by the amino acid located at the extreme C-terminus (position 0) and the amino acid at position -2. ~~, and~~ PDZ domains are classified into three groups (class I to III) based on the amino acid sequence in the interacting motif.

Some of the drug transporters which belong to the SLC superfamily have a class I PDZ binding motif (-S/T-X-Φ, Φ is a hydrophobic amino acid) at their extreme C-terminus (Table 1). Interestingly, most of the transporters with the class I PDZ motif in human and/or mouse orthologs, such as PEPT1, PEPT2, OAT4, OCT3, OCTN1, OCTN2, OATP-A and OATP-B, are expressed on apical membranes in kidney and/or small intestine (Table 1), implying that this motif could play a role as an apical localization signal (Russel *et al*., 2002). The presence of the PDZ binding motif in apically expressed drug transporters could be compatible with the idea that PDZ domain-containing proteins orchestrate plasma membrane retention and post endocytic sorting operations through interaction with membrane proteins in polarized epithelial cells (Campo *et al*., 2005). A fundamental role of the PDZ motif may also be supported by the fact that this motif is generally conserved between human and mouse, except for PEPT1 and OATP family members (Table 1). Further studies are

needed to clarify whether such difference in C-terminal sequences is relevant to differential regulation of transporters in various species. ~~Although the C-terminal sequence of human PEPT1 does not exactly fit the class I motif, the crab-eating macaque, pig, canine, sheep, rabbit, rat and mouse orthologs all have the C-terminal sequence -TXM which fits a class I motif. The status of the PDZ motif in the OATP family is more controversial, probably because human OATP orthologs do not necessarily correspond to mouse orthologs (Hagenbuch and Meier 2003). In the OATP1A family, for example, OATP-A has a class I motif (Table 1), and the C-terminus in all the Oatp1a family members in mouse (Oatp1a1/Oatp1, Oatp1a4/Oatp2, Oatp1a5/Oatp3, Oatp1a6/Oatp5) is -TKL which fits a class I motif. In the OATP2B family, on the other hand, OATP-B has a class I motif (Table 1), whereas the C-terminal sequence of Oatp2b1/Oatp9 is -QQL.~~

2-2) Requirement of C-terminal PDZ motif for intracellular localization

The presence of an apical sorting signal in the C-terminal region has been suggested for several drug transporters including MRP2. The MRP2 mutant with the last three amino acids (-TKF) deleted is predominantly localized in the basolateral membrane of MDCK cells (Harris *et al*., 2001). The deletion of 15 C-terminal amino acids results in impaired apical localization of MRP2 in HepG2 cells (Nies *et al*., 2002).

These results suggest that the C-terminal residues are essential for apical localization of MRP2. However, the MRP2 mutant with the last three amino acids deleted is targeted to plasma membranes of L1210 cells, whereas the parent construct of MRP2 is localized intracellularly (Harris *et al*., 2001), suggesting that the function of the sorting signal in transporters depends on the host cell type, possibly due to differences in the adaptor proteins interacting with the sorting signal.

~~Thus, the PDZ-binding motif in MRP2 may play a role as an apical targeting and/or retention signal. However, it should also be noted that~~ PDZ binding motifs act as a signal for targeting of at least certain types of proteins to subcellular compartments other than apical membranes. For example, basolateral sorting of ErbB receptors, potassium channel Kir 2.3 and γ-aminobutyric acid transporter BGT-1 requires a PDZ binding motif in the cytosolic domain (Campo *et al*., 2005). Thus, to understand better the physiological roles of the PDZ binding motif in drug transporters, the nature of the interacting proteins and the physiological relevance of the interactions should be fully clarified.

2-3) Protein-protein interaction at the PDZ binding motif

Until recently, information on protein-protein interactions has been quite limited for drug transporters. Yeast two-hybrid analysis first identified a specific interaction of the C-terminal cytosolic region of MRP2

with the PDZ domain-containing protein PDZK1 (also designated as Diphor-1/NaPi-Cap1/CLAMP/CAP70) (Table 2)(Kocher *et al*., 1999). Thereafter, interaction analysis by Gilser *et al*. (2003) revealed the interaction of PDZK1 with several drug transporters, including urate transporter (URAT1), OCTN1 and Oatp5. To examine the overall pattern of the interaction of drug transporters with PDZ domain-containing proteins, we have recently performed yeast two-hybrid interaction analysis of the C-terminal cytosolic region of drug transporters with four PDZ domain-containing proteins which are known to interact with other SLC superfamily members (Kato *et al*., 2004) (Table 1). This screening clearly indicated specific interactions of certain drug transporters with PDZ proteins, and all the drug transporters that showed a positive interaction have a PDZ binding motif at their C-terminus (Table 1), suggesting that these C-terminal motifs act as a signal for specific interaction. These PDZ proteins include PDZK1, PDZK2 (also named NaPiCap2, and intestinal and kidney-enriched PDZ protein, IKEPP), NHERF1 and NHERF2 (SLC9A3R2, also named E3KARP, SIP-1 and TKA-1)(Table 1, Fig. 2). The interaction was confirmed by using recombinant C-terminal regions and PDZ proteins (Gisler *et al*., 2003; Anzai *et al*., 2004; Kato *et al*., 2004, 2005).

PDZK1 and PDZK2 have four PDZ domains in its structure (Fig. 2), each PDZ domain having been proposed to bind independently to a

PDZ motif (Table 2). Yeast two-hybrid and pull-down studies using recombinant proteins have identified a number of interacting membrane proteins for each PDZ domain in PDZK1 (Table 2). Some of the membrane proteins can bind to only a single PDZ domain in PDZK1, whereas others can bind to 2 or 3 PDZ domains (Table 2, Fig. 2). For example, ~~the C-terminal region in CFTR binds to PDZ3 with high affinity (Kd = 8 nM), and to PDZ1 and PDZ4 with low affinity (Kd = 100-200 nM), but does not bind to PDZ2 (Wang *et al*., 2000) (Table 2, Fig. 2). Similarly,~~ the C-terminal domain in URAT1 binds to PDZ1 with high affinity (Kd = 2 nM), and to PDZ2 and PDZ4 with low affinity (Kd = 300-500 nM), but does not bind to PDZ3 (Anzai *et al*., 2004) (Table 2, Fig. 2). The physiological relevance of such multiple binding domains in PDZK1 for each transporter has not yet been clarified, except that the tandem PDZ domains interacting with CFTR are essential for functional modulation by PDZK1 (Wang *et al*., 2000). The interaction potential among multiple domains may allow fine functional regulation of drug transporters.

Immunohistochemical and biochemical analyses have indicated that both PDZK1 and several drug transporters are colocalized on apical membranes of renal proximal tubular epithelial cells (Gisler *et al*., 2003; Anzai *et al*., 2004; Kato *et al*., 2004, 2005). In addition, NHERF1 can also bind to NHERF1 itself, NHERF2 and PDZK1 (Gisler *et al*., 2003; Lau and Hall 2001). These results suggest that drug transporters may be localized

within a large protein complex consisting of several PDZ proteins (Gisler *et al*., 2003; Kato *et al*., 2004). Although the physiological significance of such a network should be clarified by further analysis, it may provide a structural basis for functional coupling of drug transporters. For example, PEPT2 might be localized in close proximity to NHE3, a Na^+/H^+ exchanger which is expressed on apical membranes of renal proximal tubules, because PDZK1 can bind to PEPT2 and NHE3 at different PDZ domains (Table 2, Fig. 3). Alternatively, NHE3 may bind to NHERF1, which can then interact with PEPT2 indirectly through PDZK1 (Table 2, Fig. 3). Therefore, a precise understanding of the structural organization of each transporter protein could be important for estimating membrane transport efficacy for various substrates at apical membranes in the kidney.

Protein-protein interaction and protein network formation in the kidney have been extensively studied for NHE3, and the results indicate colocalization of NHE3 with other membrane proteins. These proteins include megalin, which belongs to the LDL receptor superfamily and is involved in renal reabsorption of various compounds and polypeptides, and dipeptidyl peptidase-IV (DPPIV, also known as CD26), which is a highly specific serine protease that cleaves N-terminal dipeptides from peptides with a penultimate proline or alanine (Biemesderfer *et al*., 1999; Girardi *et al*., 2001). It should be noted however, that this network consists of distinct compartments, since NHE3 antibody can immunoprecipite DPPIV, but not

sodium-phosphate cotransporter (NPT) 2, which is also expressed on apical membranes of renal proximal tubules, and can interact with NHERF1 and PDZK1 (Gisler *et al.*, 2001) (Table 2). DPPIV and megalin can be independently co-precipitated with NHE3 by different NHE3 antibodies (Biemesderfer *et al.*, 1999; Girardi *et al.*, 2001), also supporting such distinct compartmentalization. ~~In addition,~~ Although protein-protein interaction studies have suggested possible formation of large protein complexes consisting of multiple transporters and PDZ proteins, no study has actually isolated or visualized such a complex. Thus, details of the organization of these membrane transporters remain to be clarified.

The PDZ binding signal in MRP2 also matches the protein kinase (PK) C phosphorylation site designated as [S/T]-X-[R/K]. Hegedus *et al.* (2003) reported, based on *in vitro* binding experiments, a high interaction potential with NHERF1 of the phosphorylated C-terminal peptide of MRP2. This may be compatible with the previous finding that PMA treatment resulted in mistargeting of MRP2 to the basolateral membrane in HepG2 cells (Kubitzb *et al.*, 2001). Thus, at least certain types of PDZ domain-PDZ binding motif interaction may not represent just physical interaction, but may also be involved in regulation of intracellular trafficking and/or signaling.

3. PDZ adaptor proteins involved in regulation of expression and function of transporters

3-1) PDZK1 family: Structure, expression and localization

Both PDZK1 and PDZK2 have ~~four PDZ domains in their structure (Fig. 2) and they have~~ 28% amino acid sequence identity in the case of the human orthologs. Since no other distinct structure than PDZ domains have been defined in these PDZK family proteins, these can be categorized as simple adaptors (Bezprozvanny and Maximov 2001) (Fig. 2). The primary structure of PDZK1 is highly conserved among mammalian species, with 78% identity between the human and mouse orthologs. This is also the case for PDZK2, since human PDZK2 has 81% identity with mouse NaPiCap2, which is probably the mouse ortholog for PDZK2.

PDZK1 was identified by differential display-PCR techniques as a protein which is up-regulated in rats on a low-phosphate diet, and it increased the phosphate-transporting activity of NPT2 transfected into *Xenopus laevis* oocytes (Custer *et al*., 1997). PDZK1 was also identified as a PDZ domain-containing protein which is up-regulated in several carcinoma cell lines (Kocher *et al*., 1999) or increases cell-surface expression of HDL receptor scavenger receptor class B type I (SR-B1) (Ikemoto *et al*., 2000).

On the other hand, PDZK2 was first identified as NaPiCap2 in an adult mouse kidney cDNA library by yeast two-hybrid screening as an

interacting partner for NPT2a (Gisler *et al*., 2001). Human PDZK2 was also identified by yeast two-hybrid screening as a regulator of guanylyl cyclase C, the heat-stable enterotoxin receptor (Scott *et al*., 2002). Thus, both PDZ proteins may interact with various types of cellular proteins.

In northern blot analysis, PDZK1 was detected in mouse liver, kidney, small intestine and testis, whereas PDZK2 was expressed only in kidney and small intestine (Gisler *et al*., 2001). Western blot analysis in mouse tissues revealed higher expression of PDZK1 in kidney, followed by liver and jejunum, significant expression being also observed in brain, lung and testis (Wang *et al*., 2000). In mouse kidney both PDZK1 and PDZK2 are immunostained on apical membranes exclusively in proximal tubules, and the immunostaining was uniform along the proximal tubules (S1, S2, and S3 segments) (Gisler *et al*., 2001). PDZK1 is strictly associated with the microvilli, whereas PDZK2 is predominantly located in the subapical compartment, faint immunostaining being also found throughout the cytoplasm, but not in the microvilli (Gisler *et al*., 2001).

In rabbits, mRNA and protein expression of PDZK1 is much lower in the colon than in the small intestine (Rossmann *et al*., 2005). On the other hand, in humans, gene expression of PDZK1 assessed by Northern blot analysis was observed in kidney, liver and small intestine (Wang *et al*., 2000). Northern blot analysis revealed PDZK2 mRNA expression almost exclusively in the kidney, with much lower levels in the small intestine and

colon in humans. Immunohistochemical analysis revealed localization of PDZK2 on the apical surface in cells of the crypt and villus of human ileum and colon (Scott *et al*., 2002). Overall, these two PDZK1 family members are expressed on apical membranes in both kidney and small intestine of each species, although distinct localizations may also occur and be relevant to their specific physiological roles.

3-2) NHERF family: Structure, expression and localization

NHERF1 was first purified by means of biochemical techniques and cloned as a protein kinase A-mediated regulator of NHE in rabbit renal brush border membranes, whereas NHERF2 was identified by yeast two-hybrid analysis as a protein interacting with the C-terminal cytoplasmic region of NHE3 (Weinman *et al*., 1993, 1995; Yun *et al*., 1997). NHERF1 has high homology with NHERF2 (~52% identity for the human orthologs), both having two PDZ domains at the N-terminal side (Fig. 2). In contrast to the PDZK1 family members, which have only~~four~~ tandem PDZ domains in their structure (Fig. 2), NHERF1 and NHERF2 also have an ERM (ezrin-radixin-moesin) binding domain at their C-terminal region, which can directly bind to the FERM (band four-point-one, ERM) domain located at the N-terminal region of ERM family members (Bretscher *et al*., 2000, 2002; Voltz *et al*., 2001) (Fig. 2).

In that sense, these NHERF family proteins can be categorized as complex adaptors (Bezprozvanny and Maximov 2001).

~~NHERF1 and NHERF2 can form both homo- and hetero-oligomers, and~~ Oligomerization of NHERF1 and NHERF2 is differentially regulated: NHERF1/NHERF1 association has a relatively lower affinity, but has a higher affinity in the presence of the C-terminal ligand of transmembrane proteins, and it can be affected by a constitutively phosphorylated serine residue within NHERF1, whereas NHERF2/NHERF2 association has a relatively higher affinity and is only minimally affected by interaction with the C-terminal ligand (Lau and Hall 2001). Such characteristics of the intermolecular interactions may be important for stabilization of membrane proteins including transporters, in cell-surface membranes.

In the mouse, western blot analysis revealed strong expression of NHERF1 both in kidney and small intestine, with intermediate levels being observed in liver and stomach; NHERF2 protein is expressed abundantly in the lung, but its expression in the above organs is quite low (Ingraffea *et al.*, 2002). In humans, mRNA expression, assessed by northern blot analysis, of both NHERF1 and NHERF2 was ubiquitous, except that NHERF1 mRNA was absent in lung and skeletal muscle (Yun *et al.*, 1997).

Immunohistochemical analyses of NHERF family members have revealed their apical localization in kidney and small intestine, but

localization in other subcellular compartments is also likely: in rat kidney, NHERF1 is immunostained most abundantly in the apical brush-border membrane, cytosol and, to a lesser degree, in the basolateral membranes of renal proximal tubules, but is not expressed in any other nephron structures (Wade *et al*., 2001). On the other hand, NHERF2 is strongly detected in the glomerulus and other vascular elements, and in the cortical and medullary collecting ducts, but is not clearly immunostained in proximal tubules (Wade *et al*., 2001). The predominant expression of NHERF1, but not NHERF2, in proximal tubules was also reported in mouse kidney (Ingraffea *et al*., 2002). Mouse proximal tubules express both NHERF1 and NHERF2, but NHERF1 is strongly expressed in microvilli in association with ezrin, whereas NHERF2 is weakly detected in the microvilli, but is expressed predominantly at the base of the microvilli in the vesicle-rich domain (Wade *et al*., 2003).

Immunohistochemical analysis in the rabbit small intestine revealed localization of NHERF1 on ileal brush border membranes (Kim *et al*., 2002). Localization of NHERF1 in the brush border of columnar epithelial cells in the small intestine was also found in mouse, with striking colocalization with ezrin (Ingraffea *et al*., 2002). In rat liver, NHERF1 is expressed on the apical domain of hepatocytes, whereas NHERF2 expression in hepatocytes was hardly detected (Fouassier *et al*., 2001). In mouse liver, NHERF1 is expressed in bile canaliculi of hepatocytes,

colocalizing with radixin, while expression of NHERF2 was not detected (Ingraffea *et al*., 2002).

3-3) Effect of PDZ proteins on membrane transport in vitro

Experimental findings *in vitro* have suggested that PDZK1 directly regulates the cell-surface expression and/or functional activity of various types of transporters. Custer *et al*. (1997) first reported increased phosphate transport by NPT2 in the presence of PDZK1. Wang *et al*. (2000) suggested that PDZK1 promotes intermolecular interaction of CFTRs, leading to potentiation of the chloride channel activity. The C-terminus of CFTR can interact with three of the four PDZ domains (PDZ1, PDZ3 and PDZ4) in PDZK1 (Fig. 2, Table 2, Wang *et al*., 2000). Interestingly, a stimulatory effect on channel activity was seen with a bivalent monoclonal antibody for CFTR and PDZ3-4 of PDZK1, but not with PDZ1-2, PDZ2-3 nor PDZ3, suggesting that linkage of at least two CFTR molecules via the C-terminus is necessary for the stimulatory effect (Wang *et al*., 2000). Such bivalent PDZ domain-mediated functional modulation of CFTR was also observed with NHERF1 (Raghuram *et al*., 2001).

Regarding the stabilization of drug transporters, Anzai *et al*. (2004) found that coexpression of PDZK1 increases transport capacity for urate by URAT1 in HEK293 cells, and such an increase can be explained by an increase in cell-surface expression of URAT1 induced by PDZK1.

On the other hand, transport of GlySar by PEPT2 and carnitine by OCTN2 was increased in HEK293 stably expressing PDZK1, and such an increase cannot be explained by the change in mRNA for PEPT2 or cell-surface expression of OCTN2, respectively (Kato *et al*., 2004, 2005). Coexpression of PDZK1 results in an approximately 6-fold increase in transport capacity for carnitine by OCTN2 with minimal change in transport affinity, whereas modulation of the transport function by PDZK1 is less marked for PEPT2 and URAT1 (Kato *et al*., 2004, 2005; Anzai *et al*., 2004). In all cases, transporter mutants with deletion of the last three or four amino acids at the C-terminus exhibited no increase in activity in the presence of PDZK1, suggesting that interaction of PDZK1 with the transporters is responsible for the stabilization and functional regulation of the transporters by PDZK1 (Anzai *et al*., 2004; Kato *et al*., 2004, 2005). In addition, PDZK1 is colocalized with URAT1 and OCTN2 on apical membranes in proximal tubules in kidney, and pull-down assay or immunoprecipitation also suggested the colocalization of those transporters with PDZK1 *in vivo* (Anzai *et al*., 2004; Kato *et al*., 2005). However, all the experimental evidence suggesting the modulation of drug transporters by PDZK1 has been obtained *in vitro*, except that essential role of PDZK1 in basolateral expression of Oatp1a1/Oatp1 in hepatocytes was recently demonstrated *in vivo* using PDZK1 knockout mice (Wang *et al*., 2005).

Considering that cell-surface expression and/or functional activity of transporters are directly regulated by the PDZ proteins, at least *in vitro*, endogenous expression of the PDZ proteins may affect the expression and/or function of transporters either endogenously expressed or exogenously transfected in the cell lines used for *in vitro* assays. Actually, it has been demonstrated that PDZ domain-NPT2a interaction is essential for apical localization of the transporter in LLC-pK1 cells (Hernando *et al*., 2002). As shown in Table 3, endogenous expression of the four PDZ domain-containing proteins is relatively ubiquitous in various cell lines. This may at least partially explain the apparently functional expression of drug transporters in most cell lines (CHO, LLC-pK1, HEK293, HeLa, etc) that have been usually used for *in vitro* analysis of drug transporters (Table 3). However, it is sometimes observed that transporters exogenously transfected into cell lines do not express their proper function and/or are not targeted to plasma membranes. For example, endogenous NPT2a and exogenously transfected NPT2a are properly targeted to apical membranes in OK cells, whereas NPT2 transfected into LLC-pK1 cells is mainly localized on apical membranes, but weak expression is also found in the basolateral region (Hernando *et al*., 2000). On the other hand, NPT2a transfected into MDCK cells is detected equally at basolateral and apical membranes, and that transfected into Caco2 cells does not reach the membranes, but remains in the cytoplasm (Hernando *et al*., 2000, 2001).

3-4) Gene knockout mice for assessing the physiological roles of PDZ proteins

Considering that PDZK1 can interact with various types of transporters through multiple PDZ domains in its structure and thereby regulate the expression and/or function of the membrane proteins (Fig. 2, Table 2), changes in the gene expression level of PDZK1 could possibly affect the expression and/or localization of membrane transporters, consequently affecting the membrane transport kinetics of various types of compounds. As a promising approach to analyze the physiological roles of PDZ proteins, PDZK1 gene knockout mice were first developed by Kocher *et al*. (2003a). PDZK1-deficient mice developed normally, did not display any gross phenotypic abnormalities, and exhibited apparently normal immunohistochemical localization of MRP2 and NPT2a in the kidney, probably due to functional compensation by other PDZ proteins (Kocher *et al*., 2003a). However, they exhibit an approximately two-fold elevation of plasma total cholesterol level, which is thought to result from a 95% reduction in the HDL receptor scavenger receptor class B type I (SR-B1) gene product in the liver, with only a minimal change in its mRNA level (Kocher *et al*., 2003b).

In the PDZK1 knockout mouse, expression of NPT2a is decreased under a high-Pi diet condition, with a concomitant higher urinary fractional

Pi excretion (Capuano *et al*., 2004). In the NHERF1 gene knockout mouse, on the other hand, NPT2 expression on brush border membranes in the kidney is lower than that in the wild-type mouse, NPT2 being aberrantly localized at internal sites in the renal proximal tubule cells, concomitantly with a higher urinary excretion of phosphate (Weinman *et al*., 2003a; Shenolikar *et al*., 2002). Although the NHERF1 knockout mouse exhibited normal blood electrolytes, smaller body weight than in the wild-type mouse was observed in females, and disorganized microvilli were seen in the small intestine (Morales *et al*., 2004). Thus, both NHERF1 and PDZK1 could be involved in cell-surface stabilization and/or expression of NPT2 at least under some conditions. In humans, NHERF1 is also expressed in the uppermost stratum granular layer of epidermis, and SNP in a putative RUNX1 binding site close to the NHERF1 gene is associated with psoriasis (Helms *et al*., 2003). Recent biopharmaceutical analysis has revealed functional expression of drug transporters in the skin (Li *et al*., 2005). Further analysis of the association of NHERF1 and drug transporters should improve our understanding of drug permeation in the skin.

3-5) Regulation of PDZ proteins

PDZ proteins can interact with other cellular proteins besides transporters, implying that transporter-PDZ protein interactions lead to further protein network formation. Both PDZ1 and PDZ4 in PDZK1 have

been reported to interact with MAP17 (17-kD membrane-associated protein, also known as DD96, SPAP and pKe#192), which is expressed in the proximal tubules of the kidney cortex, spermatids of the seminiferous tubules and upper stratum granulosum of normal epidermis keratinocytes (Blasco *et al*., 2003; Jaeger *et al*., 2000). Human MAP17 has 114 amino acids with a PDZ binding motif (-TPM) at the extreme C-terminus. MAP17 spans the cellular membranes twice, with both the N- and C-termini being inside the cells (Blasco *et al*., 2003). MAP17 was first identified by means of a differential display technique as a molecule highly up-regulated in human renal cell carcinoma (Kocher *et al*., 1995). MAP17 seems to exhibit specific interaction with PDZK1, since GST fusion protein only with PDZK1, but not with PDZK2 or NHERF1 can pull-down MAP17 in renal brush border membrane vesicles (Pribanic *et al*., 2003).

MAP17 is co-immunoprecipitated with PDZK1 both in liver and kidney of mouse (Silver *et al*., 2003), suggesting an interaction of the two proteins *in vivo*. MAP17 exhibited apical localization after transfection into OK cells, showing colocalization with endogenous NPT2a (Pribanic *et al*., 2003). On the other hand, PDZK1 showed a cytoplasmic distribution when transfected alone into OK cells, whereas coexpression of PDZK1 with MAP17 resulted in apical colocalization of the two proteins (Pribanic *et al*., 2003). Deletion of the last three amino acids in MAP17 shifted its subcellular localization into both apical and cytoplasmic regions, but it still

exhibited colocalization with PDZK1 (Pribanic *et al.*, 2003). Thus, localization of PDZK1 is regulated by MAP17, at least in transfectant systems with *in vitro* cell lines. On the other hand, *in vivo* overexpression of MAP17 results in the absence of PDZK1, without any effect on expression of PDZK2, in mouse liver (Silver *et al.*, 2003). In kidney, PDZK1 is equally distributed throughout the whole proximal tubules (Pribanic *et al.*, 2003), whereas MAP17 exhibited higher apical localization in S1 than S3 segments (Pribanic *et al.*, 2003). Further studies are needed to establish the physiological significance of the MAP17-PDZK1 interaction *in vivo*.

On the other hand, both NHERF1 and NHERF2 have an ERM binding domain at their C-terminal region (Fig. 2); this can bind directly to the FERM (band four-point-one, ERM) domain located at the N-terminal region in members of the ERM family (Bretscher *et al.*, 2000, 2002; Voltz *et al.*, 2001). The FERM domain can also interact directly with C-ERMAD (C-terminal ERM association domain) in ERM proteins, forming an inactive state. Activation of ERM protein is accomplished by C-terminal phosphorylation, resulting in relief of the FERM/C-ERMAD interaction and regeneration of interaction potential of FERM with NHERFs. This activation is compatible with the observation of high levels of phosphorylated ERM proteins in membrane fractions in both kidney and intestine (Morales *et al.*, 2004). The C-terminal region in ERM proteins can

interact with filamentous (F)-actin, suggesting a fundamental role in the structural organization of polarized epithelial cells by connecting F-actin to plasma membrane proteins, including transporters. Ezrin is highly expressed both in kidney and small intestine, whereas radixin is the major ERM protein in liver.

Interestingly, expression of ezrin, especially phosphorylated ezrin, on brush membranes in both kidney and small intestine is clearly down-regulated in NHERF1 gene knockout mouse, demonstrating that NHERF1 is required for the maintenance of activated ezrin on brush border membranes (Morales *et al*., 2004). In small intestine where ezrin is the only ERM protein expressed, disorganized microvilli with an increase in the number of goblet cells are observed in NHERF1 knockout mice, suggesting a physiological role of NHERF1 in apical membrane organization (Morales *et al*., 2004). In radixin knockout mouse, the serum concentration of conjugated bilirubin is increased compared with wild-type mouse, showing a similar phenotype to human conjugated hyperbilirubinemia in Dubin-Johnson syndrome (Kikuchi *et al*., 2002), which results from genetic deficiency in MRP2

4. Involvement of PPAR-α in gene expression of drug transporters and PDZ proteins

PPARα is a member of the large nuclear receptor superfamily and regulates expression of genes responsible for fatty acid β-oxidation and energy homeostasis. PPARα is highly expressed in liver, heart muscle, kidney and skeletal muscle, which have relatively high mitochondrial and peroxisomal fatty acid β-oxidation rates. PPARα agonists include fibric acid derivatives (fibrates), nonsteroidal anti-inflammatory drugs, and saturated and unsaturated fatty acids. PPARα is also activated by glucocorticoids and fasting. Expression of Mdr2mRNA was 5-fold increased in fasted wild-type mice, but increased only marginally in PPARα (-/-) mice. Fibrates have been used to treat dyslipidemia because they increase high-density lipoprotein cholesterol (HDL) and decrease triglyceride in serum (Berger *et al*., 2005; van Raalte *et al*., 2004; Goikoetxea *et al*., 2004). PPARα is the target of drugs that are effective in the treatment of metabolic disorders (atherogenesis, cardiovascular disease, type 2 diabetes, etc.). PPARα/γ dual agonists are also being developed.

Repeated administration of fibrates such as ciprofibrate and fenofibrate caused almost complete down-regulation of PDZK1 and SR-B1, and this mainly occurs via PPARα since PPARα (-/-) mice exhibited no down-regulation (Mardones *et al*., 2003). Considering that SR-B1 is the major HDL receptor in liver and its cell-surface expression is highly regulated by PDZK1, the down-regulation of SR-B1 could be at least partially due to the down-regulation of PDZK1, and could explain in part

the increase in HDL level after treatment with fibrates. However, a recent finding indicated the presence of a PDZK1-independent pathway for SR-B1 down-regulation (Lan *et al*., 2005), suggesting the presence of other molecular machinery for stabilizing SR-B1 in hepatocytes.

After treatment of mice with PPARα agonists such as clofibrate, ciprofibrate and di(2-ethylhexyl)phthalate, the mRNAs for hepatic transporters, Oatp1a1/Oatp1, Oatp2a1/PGT and Oatp2b1/Oatp9, were decreased, whereas that for Mrp3 was increased without any significant change in that for Mrp2 (Cheng *et al*., 2005; Maher *et al*., 2005). Such down-regulation of Oatp1a1/Oatp1 was previously reported at both the mRNA and protein levels in mouse liver, and such transcriptional regulation occurs via PPARα-activation (Kok *et al*., 2003). On the other hand, ciprofibrate treatment increases expression of Oatp1a1/Oatp1, with a decrease in Mrp2, as revealed by Western blot analysis of hepatic membrane fraction in rats (Chandra *et al*., 2005), suggesting species difference in PPARα activation. The efflux of a non-metabolized model substrate, 5(and 6)-carboxy-2',7'dichlorofluorescein (CDF) from liver to bile was found to be decreased in a rat liver perfusion system, and this could be compatible with the down-regulation of Mrp2 (Chandra *et al*., 2005). On the other hand, hepatic uptake of CDF is decreased after clofibrate treatment, and this cannot be simply explained by up-regulation of Oatp1a1/Oatp1 (Chandra *et al*., 2005). Both rat and mouse

Oatp1a1/Oatp1 interacts with PDZK1, and PDZK1 is responsible for sinusoidal localization of Oatp1a1/Oatp1, as revealed in PDZK1 gene knockout mouse (Wang *et al.*, 2005). Considering down-regulation of PDZK1 by treatment with fibrates (Mardones *et al.*, 2003), mislocalization of Oatp transporters by the down-regulation of PDZK1 might be involved in the change in hepatic membrane transport by the fibrate treatment.

Juvenile visceral steatosis (jvs) mice which have a hereditary deficiency of the OCTN2 gene exhibit fat deposition and mitochondrial abnormalities in the liver. The high-affinity site for uptake of carnitine was absent in hepatocytes obtained from jvs mouse (Yokogawa *et al.*, 1998). Considering that carnitine is efficiently transported by OCTN2, these results suggest that OCTN2 is functionally expressed in sinusoidal membranes of hepatocytes and is possibly involved in lipid homeostasis via transport of carnitine, which is essential for β-oxidation of fatty acids. Considering that PDZK1 is a functional modulator of OCTN2, down-regulation of PDZK1 after treatment with fibrates may affect carnitine uptake by OCTN2, leading to a change in fatty acid metabolism. Further studies are needed to clarify the pharmacological significance of the OCTN2/PDZK1 system in energy homeostasis in liver.

There are large species differences in the expression of PPARα. Human liver has less than one-tenth the levels of PPARα expression observed in mice and rats. Therefore, PPARα-mediated gene activation in

humans seems unlikely to be predicted based on the phenotype observed in rodents.

5. Conclusions

Recent progress in the analysis of protein-protein interactions has revealed that the expression and/or function of at least some types of drug transporters are regulated by PDZ domain-containing proteins. Various transporters localized at apical membranes in kidney and small intestine are thought to be incorporated into protein networks (Fig. 3). Drug transporters are categorized in several families, and often have overlapping substrate specificity and cellular localization in the body, suggesting the presence of functional redundancy. Similarly, PDZ proteins have overlapping binding specificity (Table 1) and cellular localization, and data obtained in gene knockout mice suggest the occurrence of compensation of physiological function. Considering the multiple interactions between PDZ domains and cytosolic domains of various transporters, PDZ proteins have the potential to influence membrane transport of many substrates, possibly affecting disposition of these substrates in the body. The physiological roles of PDZ proteins should be clarified by further extensive analyses.

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FIGURE LEGENDS

Fig. 1 Schematic diagram representing transporter-mediated vectorial transport of xenobiotics and nutrients in renal proximal tubules.

Fig. 2 PDZ domain-containing proteins which bind to drug transporters of the SLC superfamily

Activated ERM (ezrin-radixin-moesin) can interact with ERM binding domain (ERMB) located at the C-terminus of NHERF1 and NHERF2. ERM can also bind to protein kinase A (PKA) and β-actin.

Fig. 3 Schematic diagram representing protein-protein interactions of various types of transporters and PDZ adaptors
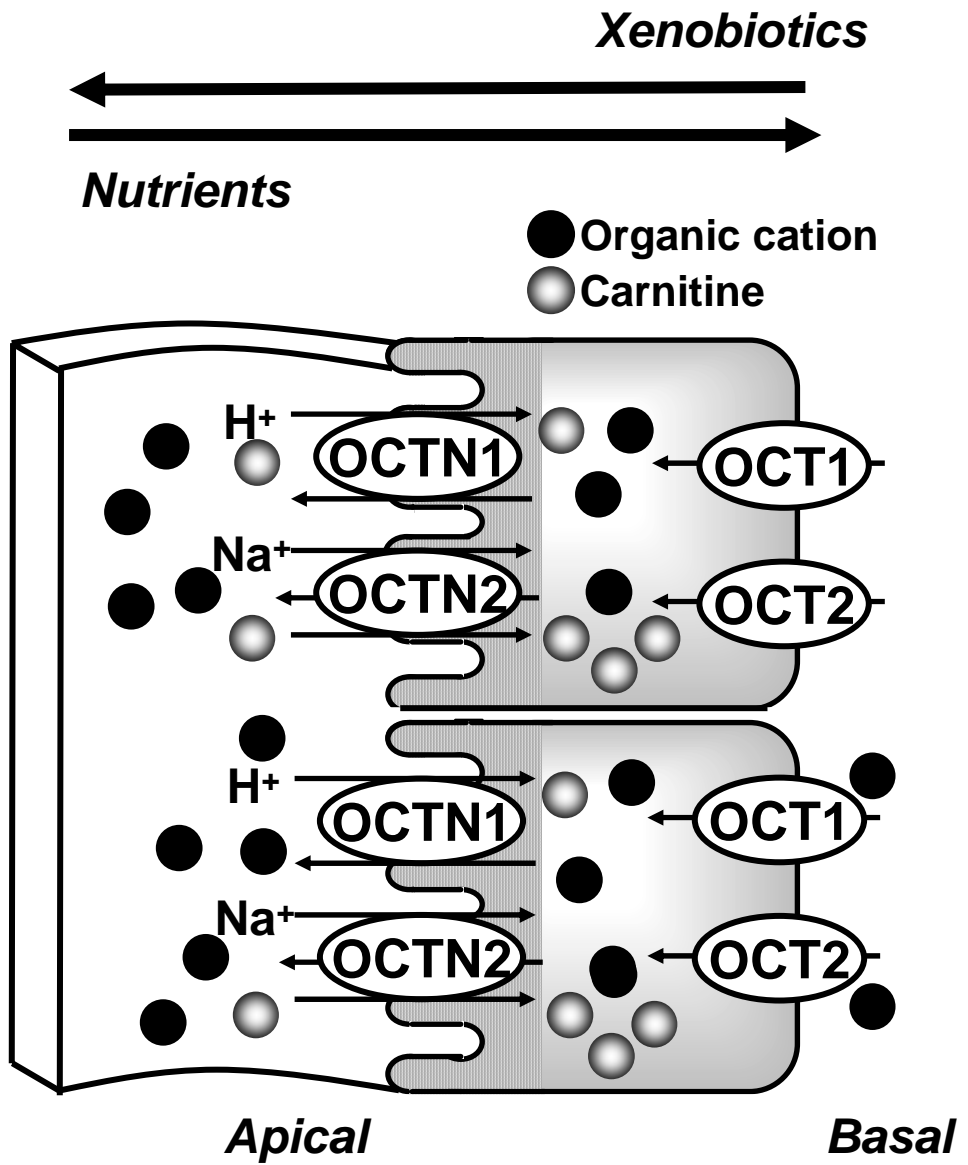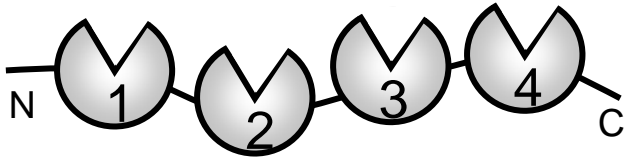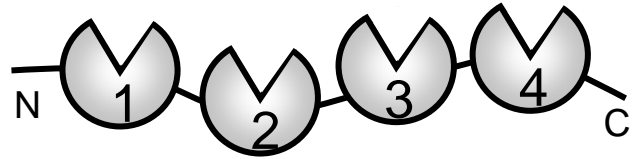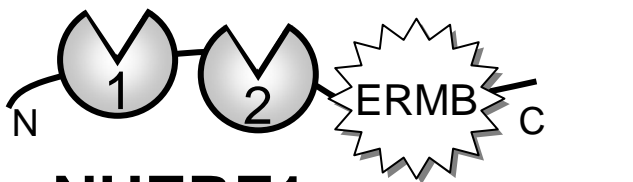
Fig. 1
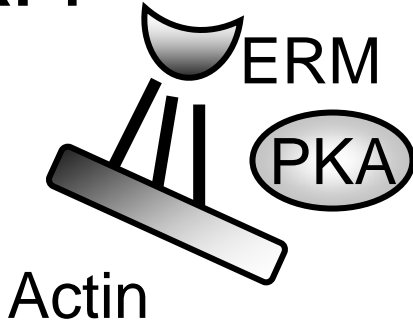
Simple Adaptors

PDZK1

PDZK2

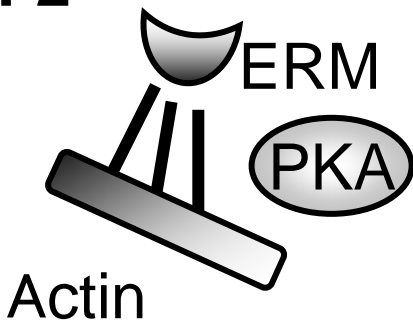**Complex Adaptors**

NHERF1

NHERF2

ERMB

ERM

PKA

Actin

Fig. 2
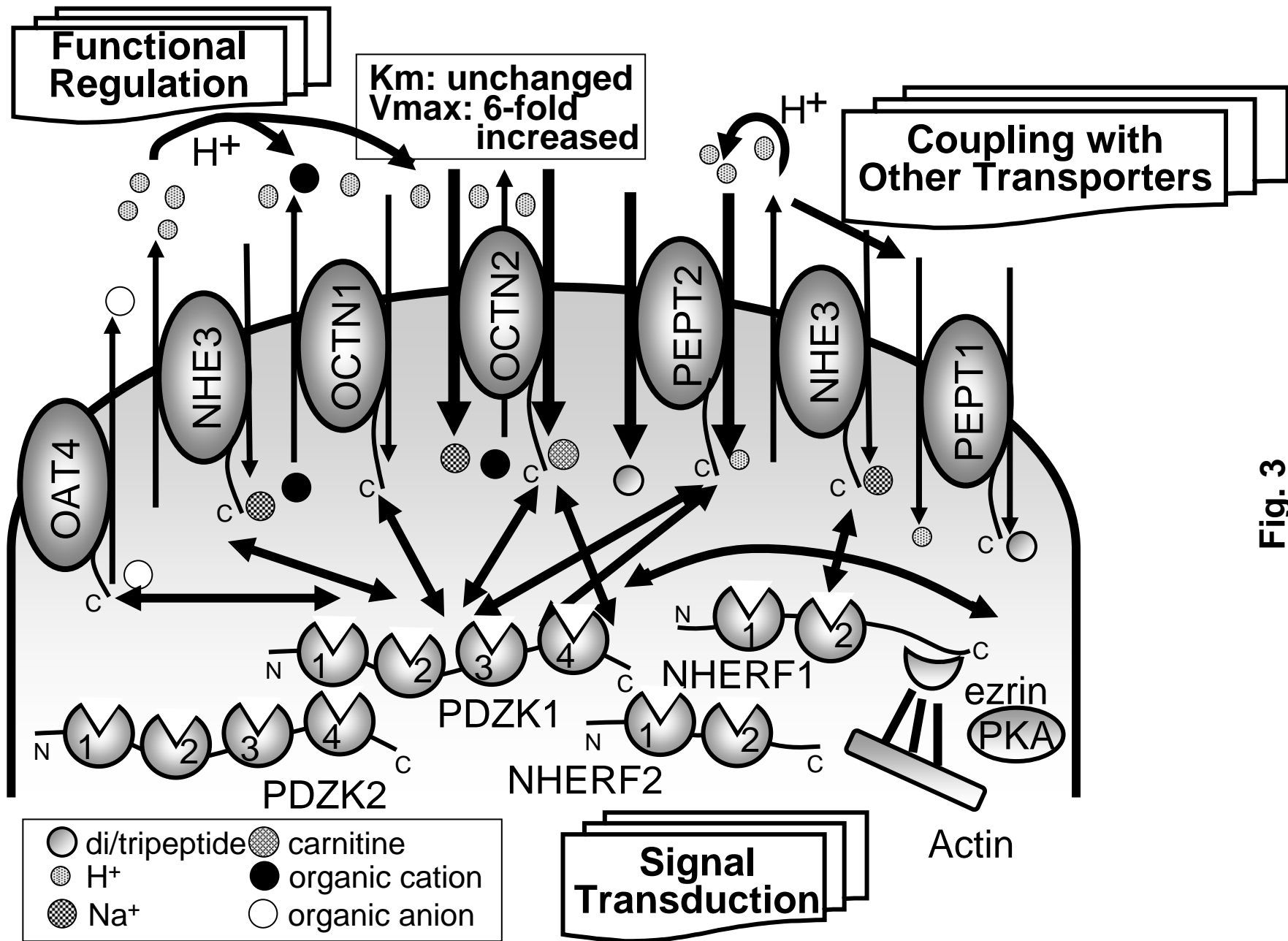
Fig. 3

Table 1. C-terminal sequence, tissue localization and protein interaction of drug transporters of the SLC superfamily in humans and mice

	C-Terminus[a]		Localization[b]			Interaction[c]			
	Human	Mouse	Kidney	Liver	Small Intestine	PDZK1	PDZK2	NHERF1	NHERF2
PEPT1	-QKQM	-Q**TN M**	Apical	Lysosome?	Apical	(-)	(+)	(-)	(-)
PEPT2	-K**TKL**	-N**TRL**	Apical	(-)	(-)	(+)	(+)	(-)	(-)
OCT1	-PSGT	-SPHT	(-)	Sinusoidal	(+)	(-)	(-)	(-)	(-)
OCT2	-IPLN	-AELS	Basolateral	(-)	(-)	(-)	(-)	(-)	(-)
OCT3	-R**S**H**L**	-T**S**D**V**	Apical	(+)	(+)	(-)	(+)	(-)	(-)
OCTN1	-I**TAF**	-I**TAF**	Apical	(+)	(+)	(+)	(+)	(+)	(+)
OCTN2	-S**TAF**	-S**TAF**	Apical	Sinusoidal	Apical	(+)	(+)	(+)	(+)
OAT1	-KNGL	-KNGL	Basolateral	(-)	(-)	(-)	(-)	(-)	(-)
OAT2	-QVQN	-TELA	Basolateral	Sinusoidal	(-)	(-)	(-)	(-)	(-)
OAT3	-LGSS	-TGGP	Basolateral	(-)	(-)	(-)	(-)	(-)	(-)
OAT4	-S**TSL**		Apical	(-)	(-)	(+)	(+)	(+)	(+)
OATP-A	-K**TKL**		(-)	(+)	Apical	(+)	(+)	(+/-)	(+/-)
OATP-B	-D**S**R**V**		(-)	Sinusoidal	Apical	(-)	(-)	(-)	(-)
OATP-C	-ETHC		(-)	Sinusoidal	(-)	(-)	(-)	(-)	(-)
OATP-D	-E**S**V**L**		(+)	(+)	(+)	(-)	(+)	(+/-)	(+/-)
OATP-E	-Q**S**S**V**		(+)	(+)	(+)	(-)	(-)	(-)	(-)
OATP-F	-E**T**Q**L**		(-)	(-)	(-)	(-)	(+/-)	(-)	(-)
OATP-8	-AAAN		(-)	Sinusoidal	(-)	(-)	(-)	(-)	(-)

a) The C-terminal sequences for human and mouse orthologs are based on NCBI reference sequences. The class I PDZ binding motif is shown in bold. The mouse ortholog for OAT4 has not been reported, and those for OATP family members are not shown due to incomplete 1:1 correspondence between human and mouse.

b) Localization observed in human and/or rodents. OAT2 is localized on basolateral and apical side of proximal tubules in human and rats, respectively.

Table 2. Interaction of each PDZ domain in PDZK1 with C-terminal domain sequences of several membrane proteins.

PDZ Domains	Proteins	Sequences[c]	References
PDZ1	BCR	−STEV	Malmberg *et al* ., 2004
	SR-B1[a]	−EAKL	Ikemoto *et al* ., 2000
	URAT1	−STQF	Anzai *et al* ., 2004
	MRP2	−STKF	Kocher *et al* ., 1999
	MAP17	−STPM	Kocher *et al* ., 1999
	NHE3	−STHM	Gisler *et al* ., 2003
	CFTR	−DTRL	Wang *et al* ., 2000
PDZ2	OCTN1	−ITAF	Gisler *et al* ., 2003
	OCTN2	−STAF	Kato *et al* ., 2005
	URAT1	−STQF	Anzai *et al* ., 2004
	PEPT2	−KTKL	Kato *et al* ., 2004
	UBC9[b]	−FAPS	Gisler *et al* ., 2003
	DRA	−ETKF	Gisler *et al* ., 2003; Rossmann *et al* ., 2005
PDZ3	NPT1	−HTRL	Gisler *et al* ., 2003
	NPT2	−ATRL	Custer *et al* ., 1997; Gisler *et al* ., 2001, 2003
	PEPT2	−KTKL	Kato *et al* ., 2004
	Oatp5	−KTKL	Gisler *et al* ., 2003
	CFEX	−VTRL	Gisler *et al* ., 2003
	DRA	−ETKF	Gisler *et al* ., 2003; Rossmann *et al* ., 2005
	CFTR	−DTRL	Wang *et al* ., 2000
	SLK[b]	−STGS	Gisler *et al* ., 2003
PDZ4	URAT1	−STQF	Gisler *et al* ., 2003; Anzai *et al* ., 2004
	OCTN1	−ITAF	Kato *et al* ., 2005
	OCTN2	−STAF	Kato *et al* ., 2005
	MAP17	−STPM	Kocher *et al* ., 1999; Gisler *et al.* , 2003
	NHERF1	−FSNL	Gisler *et al* ., 2003
	NHERF2	−FSNF	Gisler *et al* ., 2003
	CFTR	−DTRL	Wang *et al* ., 2000
	D-AKAP2/AKAP10	−STKL	Gisler *et al* ., 2003

a) PDZ1 and approximately 40 amino acids residues following PDZ1 are necessary for the interaction.
b) These proteins were found to be expressed only in the cytosol.
c) The extreme C-terminal sequences of the human orthologs of proteins interacting with PDZK1 are shown, except for the Oatp5 sequence, which is that of the mouse ortholog.