Screening of the Interaction Between Xenobiotic Transporters and PDZ Proteins

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Purpose. Xenobiotic transporters have been proposed to be involved in membrane penetration of various therapeutic agents. As little information is available on molecular mechanism of their functional regulation, we have attempted to clarify the protein-protein interactions of such transporters as a first step to identify their regulators.
Methods. Yeast two-hybrid screening was performed to examine the interaction between carboxylic terminus of various xenobiotic transporters and PDZ (PSD95, Dlg and ZO1) domain-containing proteins. The interaction and functional regulation were also evaluated in pull-down, immunoprecipitation and transport studies.
Results. Specific interaction with PDZ proteins was identified for several xenobiotic transporters including PEPT1, PEPT2, OCT3, OCTN1, OCTN2, OAT4, OATP-A, OATP-D, and OATP-F. The potent interaction was observed between PEPT2 and PDZK1, and deletion of the last four amino acids of the PEPT2 C-terminus almost completely abrogated such interaction. Recombinant PEPT2 C-terminus fusion protein can bind to purified His_6-tagged PDZK1, confirming the involvement of two of four PDZ domains within PDZK1 in the interaction. Alanine-scanning mutation in PEPT2 revealed the presence of a consensus sequence (-T-X-L) that is responsible for the PDZK1 interaction. Transfection of PDZK1 increased the uptake of glycylsarcosine by PEPT2, whereas such stimulation was not observed for PEPT2 with the last four amino acids deleted.
Conclusions. These results first identified the interaction between PDZ proteins and the cytosolic tail of various xenobiotic transporters. PDZK1 directly interacts with PEPT2, exerting functional regulation of its transporting activity. The current findings imply the localization of PEPT2 within a protein network constructed from PDZK1 and other transporter proteins.

KEY WORDS: functional regulation; PEPT2; protein interaction; transporter.

INTRODUCTION

Recent progress in molecular biopharmaceutics have suggested the essential roles of xenobiotic transporters in membrane penetration of various types of therapeutic agents (1–6). Especially, the transporters expressed on basolateral and apical membranes of epithelial cells in liver and kidney are thought to be involved in the vectorial transport of their substrates, leading to efficient excretion of endogenous and exogenous compounds from the body. Such xenobiotic transporters include members of the solute carrier (SLC) and ATP binding cassette (ABC) superfamilies. To exert precisely their physiologic functions as excretion mechanisms for xenobiotic compounds, those transporters should be targeted to appropriate regions of plasma membranes and functionally regulated via protein-protein interaction. However, despite their fundamental importance, little is known about the regulatory mechanisms of xenobiotic transporters which belong to SLC superfamily.

Xenobiotic transporters in SLC superfamily include organic cation transporter (OCT), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), and oligopeptide transporter (PEPT) families. One of the key issues in considering possible regulatory mechanisms of xenobiotic transporters is likely to be the presence of a PDZ (PSD95, Dlg, and ZO1) interacting motif in the carboxyl terminus of various transporters expressed on the apical membranes of the kidney (Fig. 1A) (7). PDZ domains interact with short C-terminal and internal peptide motifs which usually consist of three amino acids. PDZ domains are thought to play an essential role in intracellular signaling as protein-protein recognition scaffolding modules (8–10). However, limited information is available on the interaction of most xenobiotic transporters with PDZ domain-containing proteins (11).

On the other hand, several SLC superfamily proteins such as Na^+/H^+ exchanger (NHE) 3 and Na(+)/Pi cotransporter (NPT) 2a have been clarified to interact with such proteins (12–14). Those include PDZK1 (CLAMP/Diphor-1/CAP70/NaPi-Cap1), intestinal and kidney-enriched PDZ protein (IKEPP), Na^+/H^+ exchanger regulatory factor 1 (NHERF1, also called EBP50), and NHERF2 (E3KARP, SIP-1, TKA-1), all of which have two or four PDZ domains in their structures. PDZK1 is expressed on the apical membranes of kidney (11,12) and demonstrated to interact with NPT2 (12). Overexpression of the third PDZ domain in PDZK1 disturbing the apical expression of NPT2 in opossum kidney (OK) cells (15), implying the predominant role of the PDZ domain(s) in the apical localization of NPT2. Both NHERF1 and NHERF2 are also localized on the apical membranes of the kidney, NHERF1 being expressed in proximal tubules, and NHERF2 in glomerulus, collecting duct, and vascular structures (16). Co-transfection of either NHERF1 or NHERF2 affects the cAMP-mediated regulation of NHE3 in fibroblast cell lines (17,18), whereas overexpression of NHERF2, but not NHERF1, results in Ca^{2+}-dependent inhibition of NHE3 (14). The second PDZ domain and C-terminal tail of NHERF2 bind to an internal region within the C-terminus of NHE3 (13). NPT2 can also interact with both NHERF1 and NHERF2 (12), and targeted disruption of NHERF1 results in the mislocalization of NPT2 in the mouse kidney (19), suggesting the physiologic significance of the interaction.

In the current study, we investigated the interaction between the C-terminus of various xenobiotic transporters and these four PDZ proteins which have previously been reported to interact with SLC superfamily members as a first step to understand the regulatory mechanism of the transporters. Among the interaction identified in yeast two-hybrid analysis, PEPT2 was found to interact specifically with PDZK1, this interaction being mediated by second and third PDZ domains

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ABBREVIATIONS: 3-AT, 3-aminotriazole; GST, glutathione S-transferase; IKEPP, intestinal and kidney-enriched PDZ protein; NHERF, Na^+/H^+ exchanger regulatory factor; PEPT2, peptide transporter 2; SLC, solute carrier.

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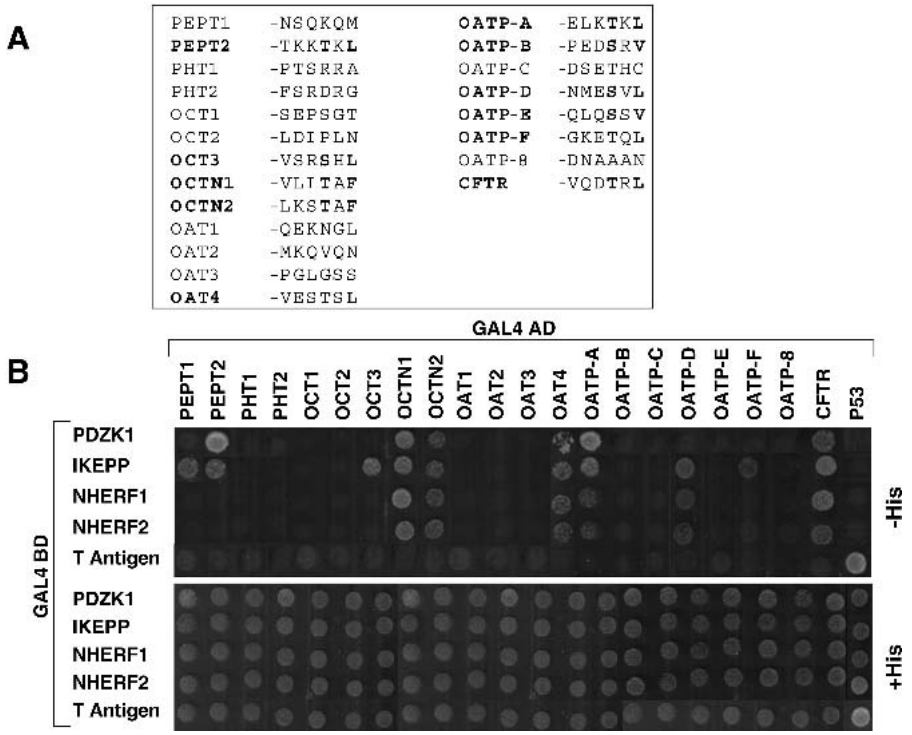

Fig. 1. Allignment of the carboxyl-terminus of xenobiotic transporters (A) and yeast two-hybrid screening for the interaction between the carboxyl-terminus of xenobiotic transporters and PDZ proteins (B). In panel A, the C-terminal sequences of several transporters are compared. The sequences consistent with a Class I PDZ domain binding motif were written in bold. In panel B, yeast cells were co-transformed with plasmids encoding each of four PDZ proteins (subcloned as a fusion protein with GAL4AD into pGADT7 vector) and the C-terminus of xenobiotic transporters or CFTR (subcloned as a fusion protein with GAL4BD in pGBKT7). T antigen and P53 were used in the control experiment. Interactions were indicated by growth on agar plates made with medium without histidine (-His). To minimize nonspecific interaction with T antigen, 3-aminotriazole (3-AT) was included in medium (1 mM for OCTN1, OAT2, OAT4, OATP-A and OATP-E; 3 mM for OAT1 and OATP-D; 6 mM for OAT3).

in PDZK1 and the C-terminus of PEPT2. The present findings have extended such PDZ domain-mediated interaction to drug and peptide transporters, suggesting the possible physiologic significance of such scaffolding proteins in the function and/or localization of the transporters.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibody for PEPT2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antibody 9E10 (c-myc) was from Covance Inc. (Princeton, NJ). The myc epitope sequence (MEEQKLISEEDL) was first introduced into the BamHI site of pcDNA3 (Invitrogen, San Diego, CA, USA) plasmid. cDNA clones encoding PDZK1, NHERF1 and NHERF2 were obtained from Invitrogen, while cDNA for IKEPP was cloned by PCR amplification (Advantage2, Clontech, Palo Alto, CA, USA) using cDNA obtained from Caco2 cells as a template. cDNA fragments encoding a single PDZ domain or each combination of two sequential PDZ domains (listed below) were obtained by PCR amplification using BamHI and XhoI sites: aa1-101 for PDZ1, aa 119-221 for PDZ2, aa 230-351 for PDZ3, aa365-519 for PDZ4, aa1-221 for PDZ1-2, aa119-351 for PDZ2-3 and aa230-519 for PDZ3-4. All these cDNAs were subcloned into pGADT7 (Clontech), pET-30a(+) (Novagen, Madison, WI, USA), and myc/pcDNA3 plasmids within BamHI/XhoI for NHERF1 and PDZK1, EcoRI/SalI for NHERF2, and EcoRI/XhoI for IKEPP. The C-terminal 41, 37, 22, 20, 41, 41, 39,44, 48, 59, 36, 50, 38, 47, 36, 44, 62, 33, 48, 55, and 360 amino acids of human PEPT1, PEPT2, PHT1, PHT2, OCT1, OCT2, OCT3, OCTN1, OCTN2, OAT1, OAT2, OAT3, OAT4, OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-F, OATP-8, and cystic fibrosis transmembrane conductance regulator protein (CFTR) was amplified by PCR and subcloned into both pGBKT7 (Clontech) and pGEX6P-1 (Amersham Biosciences, Buckinghamshire, UK) plasmids between SmaI (for OATP-8) or EcoRI (for others) and SalI sites. The full sequence for all the inserts was verified.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed as described previously (20). Briefly, yeast cells (AH109 strain) were co-transformed with pGBKT7(TRP) encoding GAL4bd fused to the PEPT2 C-terminus, and pGADT7(LEU2) vector encoding GAL4ad fused to different PDZ domain constructs. Co-transformed cells were further cultured on plates lacking leucine and tryptophan, with or without histidine.

Binding Assays

Glutathione S-transferase (GST) and His_6 fusion proteins were obtained from *E. coli* (BL-21 strain) transformed with pGEX6P-1 and pET-30a(+) constructs, respectively. GST-PEPT2 C-terminus fusion proteins (100 μg) were incubated with His_6-PDZ domains and glutathione-Sepharose 4B at 4°C for 3 h in PBS (1 ml) supplemented with 0.05% Triton X-100, 0.5 mM dithiothreitol and protease inhibitors (Complete EDTA-free, Roche Diagnostics, Basel, Switzerland) (20). The suspension was then washed twice with ice-cold PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with HRP-conjugated antibody to the His_5 tag (Qiagen, Valencia, CA, USA) using polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA). The protein was detected with the ECL-plus Western-blotting detection system (Amersham Biosciences). The GST fusion protein added to the reaction mixture was checked with Ponceau red and was almost comparable between each GST fusion protein (data not shown).

Pull-down Experiments Using Recombinant GST Fusion Proteins

HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37°C and 5% CO_2. After 24 h cultivation of HEK293 cells in 15-cm dishes, each cDNA construct was transfected by adding 20 μg of the plasmid DNA encoding myc-tagged PDZ proteins according to the calcium phosphate precipitation method (21). At 48 h after transfection, the HEK293 cells were washed twice with PBS and collected with a rubber policeman, followed by centrifugation. The supernatant was removed by aspiration, and the pellet was solubilized in RIPA-Y buffer containing 1% NONIDET P-40, 75 mM NaCl, 50 mM Tris-HCl (pH 7.5) and protease inhibitors. The cell lysate (500 μl) was then incubated with GST-PEPT2 (100 μg) and glutathione-Sepharose 4B at 4°C for 3 h, followed by washing three times with ice-cold PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-c-myc antibody. The membrane was then incubated with HRP-linked donkey anti-mouse IgG as the secondary antibody (Amersham Biosciences), and the protein was detected with the ECL-plus system.

Stable Expression of PDZK1 in HEK293 Cells and Immunoprecipitation

HEK293 cells were transfected with myc/pcDNA3 constructs containing full-length PDZK1, and stably transfected cells were selected by adding G418 (Invitrogen) to the culture medium. Two weeks after the transfection, positive clones (PDZK1/HEK293 cells) were selected by Western blot and immunohistochemical analysis. The pcDNA3 construct containing full-length PEPT2 was then transiently transfected according to the calcium phosphate precipitation method (21), and cell lysate was obtained at 48 h after transfection. C-myc antibody prebound to Protein G Sepharose (Amersham) was then added to the obtained lysate and the mixture was incubated at 4°C for 4 h, followed by centrifugation and washing three times with PBS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-PEPT2 or c-myc antibodies.

Transport Studies

At 48 h after transfection of full-length PEPT2 into HEK293 or PDZK1/HEK293 cells, the cells were harvested and suspended in transport medium (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM MES, pH 6.0). The cell suspension system was used for the uptake experiment in the present study to minimize the experimental variability possibly due to the incomplete transfection efficiency in calcium phosphate precipitation method. The cell suspension and the transport medium containing 250 nM (25 nCi/ml) of [^{3}H]glycylsarcosine (GlySar, 0.1 mCi/μmol, Moravek, Brea, CA, USA) were mixed to initiate the transport reaction. At the designated times, 200 μl aliquots of the mixture were withdrawn, and the cells 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 Chemical Industries, Osaka, Japan) with a density of 1.03 on top of 3 M KOH solution. After solubilization of each cell pellet in KOH, the cell lysate was neutralized with HCl. Then, the associated radioactivity was measured by using a liquid scintillation counter with Clearsol-1 (Nacalai Tesque, Kyoto, Japan) as a liquid scintillation fluid. Cellular protein content was determined using the Bradford method with a protein assay kit (Bio-Rad, Hercules, CA, USA). All the raw data expressed as pmol/mg protein were normalized by the medium concentraion of the substrate (pmol/μl) and represented as C/M ratio (μl/mg protein).

Determination of mRNA Expression by Real-time PCR

At 48 h after transfection of PEPT2, total RNAs were extracted, and cDNA was then synthesized as described previously (22). Quantification of the mRNA coding for PEPT2 was performed using LightCycler technology (Roche Diagnostics) (22). The PEPT2 forward and reverse primers were 5′- GCCATTGCTGACTCGTGGTT-3′ and 5′-TGTGTACCACTTGTCCTCCC-3′, respectively, which produce a 124-bp amplicon. The PCR cycle was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 0 s (followed by an immediate change into the next temperature) and combined annealing-extension at 59°C for 0 s and 72°C for 5 s, followed by fluorescence emission reading at 78°C for 10 s. The quantification value was calculated from the average values of three determinations of the three different dilutions.

RESULTS

Screening of the Interaction Between PDZ Proteins and Various Xenobiotic Transporters

Yeast two-hybrid analysis was first performed to evaluate the interaction of the C-terminus of various xenobiotic transporters, including PEPT, OAT, OCT, OCTN, and OATP families, and several PDZ domain-containing proteins which have been reported to interact with other members of the SLC superfamily (Fig. 1B). Interactions were indicated by

growth of yeast cells on agar plates in the absence of histidine (-His). The C-terminus of OCTN1, OCTN2, OAT4, and OATP-A interacted with all the four PDZ proteins examined (Fig. 1B). The C-terminus of CFTR, which was previously demonstrated to interact with NHERF1 and NHERF2 (23), PDZK1 (24), and IKEPP (25), also interacted with all the four PDZ proteins (Fig. 1B). The C-terminus of OATP-D interacted with NHERF1, NHERF2 and IKEPP (Fig. 1B). Positive interaction was also found between PEPT1 and IKEPP, PEPT2 and PDZK1, PEPT2 and IKEPP, OCT3 and IKEPP, OATP-F and IKEPP (Fig. 1B). No interaction was detected between T antigen and C-terminus of transporters nor the PDZ proteins and P53 (Fig. 1B), assuring the specificity of this assay system. In addition, growth of yeast cells in the presence of histidine was almost comparable for all the assays examined (Fig. 1B).

PEPT2 C-terminus Can Specifically Bind to PDZK1

Because the interaction between PEPT2 and PDZK1 was most remarkable in yeast two-hybrid analysis (Fig. 1B), we further attempted to examine the specificity of such interaction. The interaction with PDZK1 was not observed for the PEPT2 C-terminus with the last four amino acids deleted (Fig. 2A). In addition, the interaction of the PEPT2 C-terminus with PDZK1 was also confirmed in a pull-down study, in which the lysate of HEK293 cells transfected with myc-tagged PDZK1 was incubated with GST fusion protein with the PEPT2 C-terminus, followed by precipitation with glutathione Sepharose (Fig. 3A). The apparent molecular size of PDZK1 (~70 kD, Fig. 3A) was close to that previously observed in mouse renal brush border membrane vesicles (11) whereas that of IKEPP (~70 kD, Fig. 3A) was slightly different from that observed in Caco-2 cells (~60 kD) (26). The apparent size of NHERF1 was slightly higher than that of NHERF2 (Fig. 3A), both bands being 50–60 kD. This was compatible with the difference in that observed in PS120 cells transfected with NHERF1 or NHERF2, showing immunoreactive bands at 50–60 kD (16). The interaction required the last four amino acids in PEPT2, as their deletion resulted in almost complete loss of the interaction (Fig. 3B). In this assay, interaction with PEPT2 was observed for PDZK1, but not for IKEPP, NHERF1 or NHERF2, suggesting that the interaction is specific at least for PDZK1 (Fig. 3). On the other hand, CFTR C-terminus interacts with both PDZK1 and NHERF1, in agreement with previous reports (24,27).

To examine whether the interaction is direct or not, recombinant His_6-tagged PDZK1 was purified and incubated with GST fused with PEPT2 C-terminus (Figs. 3C and 3D). His_6-PDZK1 bound to the GST-PEPT2 fusion protein, and deletion of the last four amino acids again resulted in a marked decrease of the binding (Fig. 3D). Each amino acid was then replaced with Ala to identify the motif responsible for the interaction (Fig. 3C). Replacement of the last (Leu) or third last (Thr) amino acid with Ala resulted in a remarkable decrease in the interaction, whereas binding was only partially decreased by replacement of the second last amino acid (Lys), and replacement of the fourth last amino acid (Lys) had little effect (Fig. 3C). Thus, the motif responsible for the interaction is -TXL at the C-terminus.

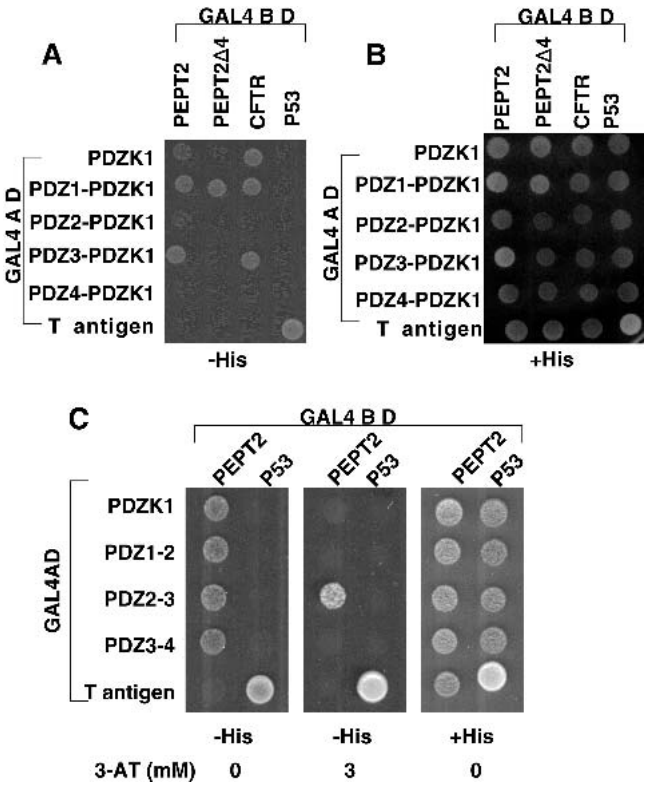

Fig. 2. Yeast two-hybrid analysis of the interaction between the carboxyl-terminus of PEPT2 and PDZK1 In panels A and B, yeast cells were co-transformed with plasmids encoding PDZK1 or each PDZ domain in PDZK1 (subcloned as a fusion protein with GAL4AD into pGADT7 vector) and the C-terminus of PEPT2, its four amino acids–deleted mutant (PEPT2Δ4) or CFTR (subcloned as a fusion protein with GAL4BD in pGBKT7). Interactions were indicated by growth of yeast cells on agar plates in the absence of histidine (panel A). The growth of yeast cells were also checked in the presence of histidine (panel B). T antigen and P53 were used in the control experiment. In panel C, each combination of two sequential PDZ domains, such as PDZ1-2, PDZ2-3 and PDZ3-4, was subcloned as a fusion protein with GAL4AD, and similar experiments were performed using 3-AT included in medium at the indicated concentration.

Multiple PDZ Domains in PDZK1 Are Responsible for the Interaction with PEPT2 C-terminus

PDZK1 has four PDZ domains in its structure, each of them being believed to interact with cytosolic tails of transmembrane proteins. To identify PDZ domains responsible for the interaction with PEPT2, yeast-two hybrid analysis was performed for each PDZ domain in PDZK1 (Fig. 2). The growth in the absence of histidine was observed for PDZ1, PDZ2, PDZ3, PDZ1-2, PDZ2-3 and PDZ3-4 (Figs. 2A and 2C). In the presence of 3-aminotriazole (3-AT) which is a competitive inhibitor of the yeast HIS3 protein and used to inhibit low levels of His3p expression, the growth was observed only for PDZ2-3 (Fig. 2C).

Next, His_6-tagged protein was purified for each PDZ domain (PDZ1 to PDZ4), and the interaction with the GST fusion with PEPT2 C-terminus was examined (Fig. 3D). His_6-tagged PDZ2 and PDZ3 bound to the GST fusion with PEPT2 C-terminus, whereas binding was not observed for His_6-tagged PDZ4 (Fig. 3D). The interaction with PDZ2 and PDZ3 was not observed for PEPT2 C-terminus with the last

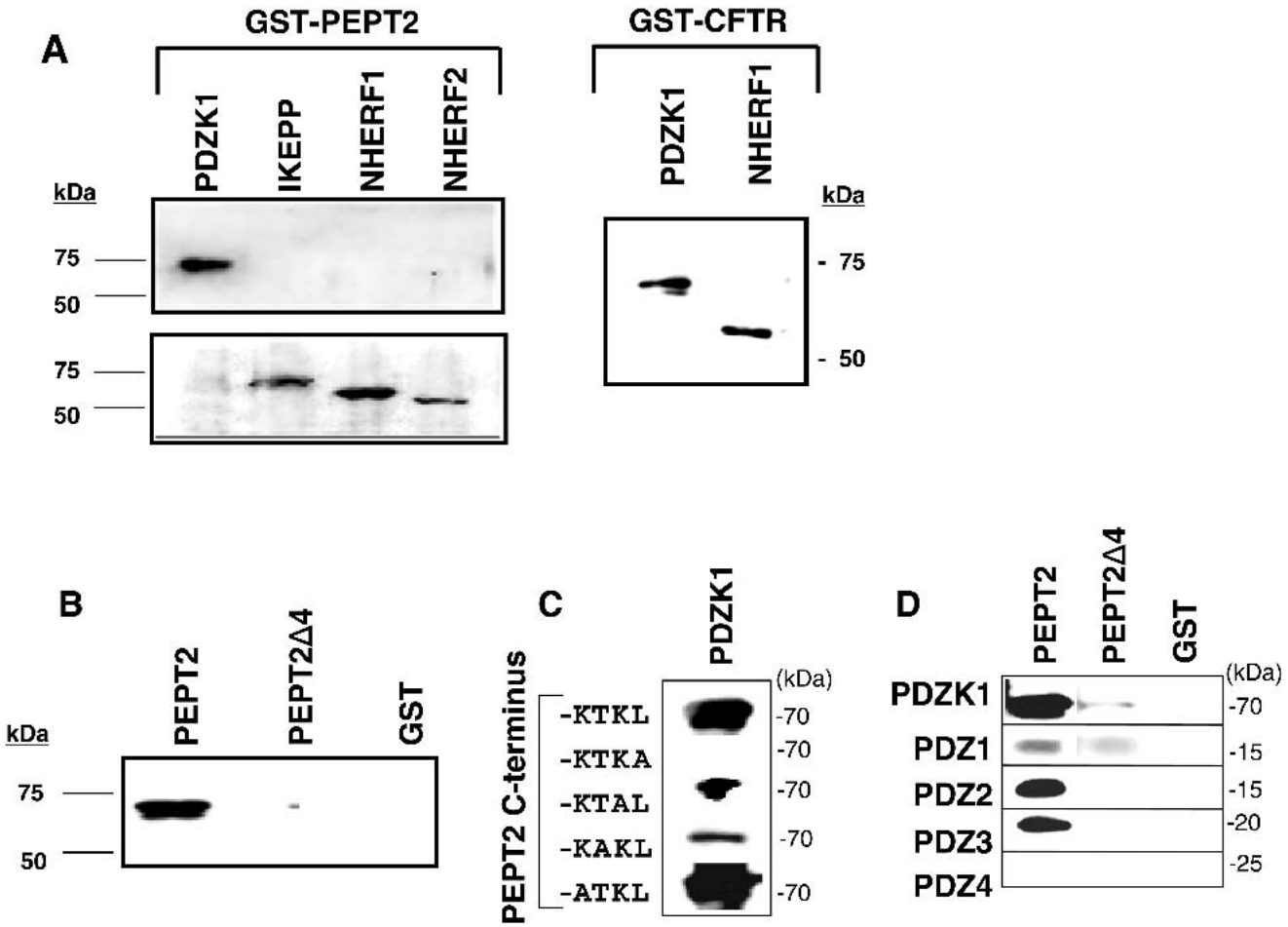

Fig. 3. Pull-down for characterization of the interaction between the PEPT2 C-terminus and PDZK1. In panel A, HEK293 cells were transiently transfected with myc constructs containing full-length PDZK1, IKEPP, NHERF1, or NHERF2, solubilized, and subjected to pull-down analysis with GST fusion protein with C-terminus of PEPT2 or CFTR. The precipitated (interacted) materials (upper panel) or 40 μg of total cell lysates (lower panel) were then analyzed by Western blotting using c-myc antibody. Note that expression of PDZK1 cannot be detected for total cell lysates, possible due to its low expression in HEK293 cells.In panel B, the lysate for the cells transfected with myc-tagged PDZK1 was subjected to pull-down analysis using GST fusion protein with the C-terminus of PEPT2 (hPEPT2), its mutant with the C-terminal four amino acids deleted (hPEPT2Δ4), or GST alone (GST). The precipitated materials were subsequently analyzed by Western blotting using c-myc antibody.In panel C, His_6-tagged PDZK1 was incubated with GST fusion protein with PEPT2 C-terminus or a mutant in which one of the last four amino acids was replaced with Ala. The interacted His_6-tagged PDZK1 was captured by glutathione Sepharose, and precipitated materials were subjected to SDS-PAGE and subsequent Western blot analysis using anti His5 antibody. In panel D, GST fusion protein with PEPT2 C-terminus, or that with the last four amino acids deleted (PEPT2Δ4), or GST alone was incubated with His_6-tagged PDZK1 or each PDZ domain, and interacted His_6-tagged protein was detected by Western blot analysis using anti-His_5 antibody. Due to the high nonspecific binding, the incubation with His_6-tagged PDZ1 was performed in the presence of 0.2% Triton X-100.

four amino acids deleted (Fig. 3D). Weak interaction was observed between His_6-tagged PDZ1 and PEPT2 (Fig. 3D), but this interaction was still observed for PEPT2 C-terminus with the last four amino acids deleted (Fig. 3D).

Co-immunoprecipitation of Full-length PEPT2 and PDZK1 in HEK293 Cells

To demonstrate that the interaction with PDZK1 can be observed for full-length PEPT2, immunoprecipitation was performed for HEK293 cells expressing both proteins. HEK293 cells transfected with both myc-tagged PDZK1 and full-length PEPT2 were solubilized and immunoprecipitated with c-myc antibody, affording a band close to 100 kDa which was immunoreactive with PEPT2 antibody (Fig. 4A). Immunoprecipitation also exhibited the 70-kDa protein that was immunoreactive with c-myc antibody (Fig. 4B). On the other hand, such an immunoreactive band was not observed when PEPT2 with the last four amino acids deleted was transfected, or when PEPT2 was not transfected (Fig. 4A).

Functional Regulation of PEPT2 by PDZK1

To obtain an insight into physiologic significance of the specific interaction between PEPT2 and PDZK1, effect of PDZK1 on transport activity of PEPT2 was examined (Fig. 5). For such purpose, PEPT2 was transiently transfected into HEK293 cells or those stably expressing PDZK1 (PDZK1/HEK293 cells). Time-dependent uptake of GlySar by PEPT2 was observed in both cell lines, initial slope of the uptake being 2.7 times higher in PDZK1/HEK293 cells than HEK293 cells (Fig. 5A). The uptake of GlySar by PEPT2 with the last

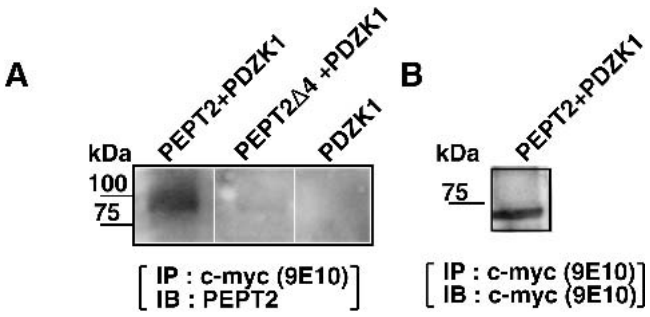

Fig. 4. Co-immunoprecipitation of PDZK1 and full-length PEPT2. PDZK1/HEK293 cells were transiently transfected with full-length PEPT2, PEPT2 with the last four amino acids deleted or vector alone. Cells were solubilized and immunoprecipitated (IP) with c-myc antibody. Western blotting (IB) was then performed using PEPT2 antibody (A) or c-myc antibody (B).

four amino acids deleted (PEPT2Δ4) in PDZK1/HEK293 cells was almost similar to that in HEK293 cells (Fig. 5B), suggesting that the enhancement effect is caused via interaction between PDZK1 and C-terminus of PEPT2. We also examined the mRNA copy number of PEPT2 both in HEK293/PDZK1 and HEK293 cells by real-time quantitative PCR analysis. PEPT2 mRNA expression was $3.34 \pm 0.05 \times 10^4$ and $5.84 \pm 0.14 \times 10^4$ copies/ng total RNA in HEK293/PDZK1 and HEK293 cells, respectively.

DISCUSSION

Various studies have suggested the involvement of many types of xenobiotic transporters in drug absorption, disposition and excretion in the body (1–6). However, information on the mechanism(s) regulating their function and/or localization is quite limited. Saito *et al.* (28) identified PEPT1 regulatory splice variant (PEPT1-RF) which alters the pH-dependence of the transport activity of PEPT1, although details of its interaction with PEPT1 are still unknown. To identify candidate protein regulatory factors of each transporter, the protein-protein interactions need to be clarified. It has been proposed that PDZ interacting motif is present in the C-terminus of various transporters expressed on the apical membranes of the kidney (7). However, there was little evidence to demonstrate the protein interactions for xenobiotic transporters in SLC superfamily although recent findings obtained by Gisler *et al.* (11) suggest the interaction of OCTN1 with NHERF1 and PDZK1. The yeast two-hybrid screening performed in the present study (Fig. 1B) provides the first evidence suggesting the possible interaction of a certain types of xenobiotic transporters with PDZ domain-containing proteins. The positive interaction with PDZ proteins was observed for PEPT1, PEPT2, OCT3, OCTN1, OCTN2, OAT4, OATP-A, OATP-D, and OATP-F (Fig. 1B). The C-terminus of all of these transporters has a class I PDZ domain binding motif (-S/T-X-Φ, Φ is a hydrophobic amino acid, Fig. 1A), suggesting that this motif may act as a signal for interacting with PDZ proteins. However, the present findings also imply that the interaction may specifically occur in a certain combinations of PDZ proteins and transporters, such combinations including PDZK1 and/or IKEPP with PEPT1, PEPT2, OCT3, and OATP-F (Fig. 1B). The C-terminal sequence of both PEPT2 and OATP-A is -T-K-L (Fig. 1A), but the binding specificity of OATP-A is more broad (Fig. 1B). Thus, PDZ-transporter interaction cannot be simply accounted for by the presence of PDZ motif at the C-terminus, and further analysis is required to clarify the structural basis for such specific interaction.

In the yeast two-hybrid analysis, the interaction of PEPT2 with PDZK1 was remarkable (Fig. 1B), leading to the further analysis for characterization of such interaction. A specific interaction between PEPT2 and PDZK1 was suggested based on at least four experimental approaches, that is, the yeast two-hybrid system (Figs. 1B and 2A), pull-down studies with lysate of PDZK1-expressing cells (Figs. 3A and 3B), binding studies using purified recombinant fusion proteins (Figs. 3C and 3D) and co-immunoprecipitation in cells expressing both proteins (Fig. 4). The deletion of the last four amino acids of the PEPT2 C-terminus resulted in a decrease in the interaction in all four experiments (Figs. 1–4), suggesting that these four amino acids (-KTKL) are critical for the interaction with PDZK1. This is compatible with previous findings that direct interaction between PDZ domain and its peptide ligand mainly occurs at the C-terminus of the ligand (9). In addition, the effect of the replacement of each amino acid with Ala on the interaction differed, and it appeared that the last and third last moieties are most important (Fig. 3A). Thus, the motif in the PEPT2 C-terminus responsible for PDZK1 interaction can be written as "-T-X-L", which is a class I PDZ domain binding motif. It should be noted that such motif in PEPT2 was conserved between human (-TKKTKL), rats (-TKNTRL), and mouse (-TKNTRL), supporting the importance of the C-terminus. Interestingly, the C-terminus of CFTR is also categorized as a class I motif, but the replacement of the second last amino acid Arg with other ones was reported to decrease the affinity for NHERF1 by 2- to 10-fold (23). This can be explained by the fact that the guanido group of Arg forms two salt bridges and two hydrogen bonds with PDZ1 of NHERF1 (29). PEPT2 also has a conserved cationic amino acid (Arg or Lys) at that position, which seems to be partially involved in the interaction (Fig. 3C).

The oligopeptide transporter PEPT2 mediates the uphill transport of di- and tripeptides and some peptide-mimetics into a variety of cells (30,31). In rat kidney, PEPT2 is localized on apical membranes of S2 and S3 segments (32). Targeted disruption of the PEPT2 gene in mice results in a decrease in renal accumulation of fluorophore-labeled and radiolabeled dipeptides (33), supporting that the possible physiologic roles of this transporter include the reabsorption of substrates. The identification of specific interaction of PEPT2 with PDZK1 implies the need for further investigation to clarify its functional relevance. Coexpression of PDZK1 increases the transport activity of PEPT2, and the increase in the initial slope of transport cannot be fully accounted for by the difference in mRNA expression of PEPT2 (Fig. 5). Because such increase in transport function also depends on the last four amino acids in PEPT2 C-terminus, the effect of PDZK1 is at least partially post-transcriptional one and possibly results from the direct interaction with PEPT2. PDZK1 is expressed on the apical membranes of renal tubular cells (11,12) and is thought to be a major scaffold protein interacting with various types of membrane transporters, including NHE3 and NPT2 (11,34). Since PEPT2 is the renal apical transporter, the interaction with PDZK1 may indicate any physiologic signifi-

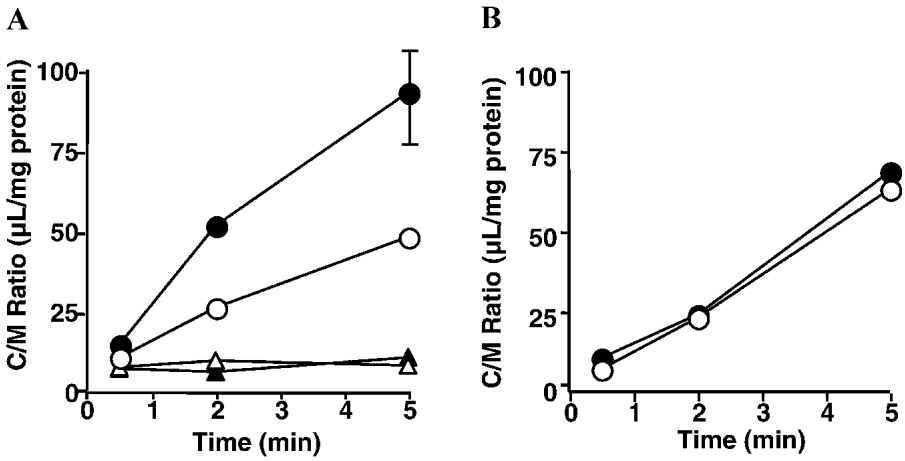

Fig. 5. Effect of PDZK1 on [^{3}H]GlySar Uptake by PEPT2 (A) or PEPT2Δ4 (B). In panel A, HEK293 (○, △) or PDZK1/HEK293 (●, ▲) cells were transfected with full-length PEPT2 (○, ●) or vector alone (△, ▲), and uptake of [^{3}H]GlySar was measured. In Panel B, HEK293 (○) or PDZK1/HEK293 (●) cells were transfected with PEPT2 with the last four amino acids deleted, and uptake of [^{3}H]GlySar was measured. Data represent mean ± SD of three determinations from one experiment. The vertical bar was not shown when the SD value was smaller than the symbol. Statistical difference ($p < 0.05$) was assessed by the Student's *t* test between HEK293 and PDZK1/HEK293 cells and observed for the uptake at 2 and 5 min by PEPT2. Similar results were obtained from other two independent experiments.

cance of such interaction. Interestingly, CFTR can also bind to the multiple PDZ domains both in PDZK1 and NHERF1, and such multiple interaction is proposed to be involved in the channel activity, probably mediated by the formation of dimer proteins (24,27,35). Therefore, there could be some physiologic relevance to the finding that PEPT2 can also interact with multiple PDZ domains (Figs. 1 and 3). The interaction of NHE3 with NHERFs, which also interact with PDZK1 (11,36), may imply another possibility for the physiologic significance of PEPT2-PDZK1 interaction. NHE3 is expressed on the apical membranes of renal tubular cells and mediates the exchange of Na^+ and H^+ across plasma membrane (37). This activity could be important for the maintenance of acid-base balance and NaCl homeostasis. Since PEPT2 is a proton-coupled transporter for oligopeptides and therapeutic agents, the physical interaction between PEPT2, NHE3, and PDZ proteins may improve the efficiency of the transfer of driving force for PEPT2 activity. A functional interaction between another peptide transporter PEPT1 and NHE3 has been conclusively shown in human intestinal epithelial cells (38). Kennedy *et al.* (39) proposed that optimal absorptive transport of GlySar is dependent on functional Na^+/H^+ exchanger activity, and that the reliance of a dipeptide transporter upon a Na^+-dependent intracellular pH regulatory mechanism may partly explain the apparent Na^+-dependence of high-affinity dipeptide uptake observed in PEPT2-expressing astrocytes (40). Thus, the present finding on protein-protein interaction may support, as a scaffolding mechanism, such functional coupling between H^+/oligopeptide transporter and Na^+/H^+ exchanger.

On the other hand, recent evidence suggests that expression and/or stability of PDZK1 requires another protein, MAP17, which was documented to be upregulated in various human carcinomas and interacts with PDZK1 (41). Transfection of both MAP17 and PDZK1 into OK cells resulted in the apical expression of PDZK1, whereas no expression of PDZK1 was observed after the transfection of PDZK1 alone (41). The unstable nature of PDZK1 was observed here in HEK293 cells, since the Western blot analysis for the lysates of HEK293 cells transiently transfected with myc-tagged PDZK1 failed to detect the expression of PDZK1 (Fig. 2A). Nevertheless, this cell line was chosen for the analysis of protein interaction analysis in the present study mainly because calcium phosphate precipitation method is applicable in HEK293 cells for the analysis of the expression and function of variety types of transporters. Our preliminary analysis using RT-PCR indicated no gene expression of PDZK1 in HEK293 cells. This may also be an advantage for the analysis of the effect of PDZK1. However, considering the possible regulation of PDZK1 by other factors, the function and/or expression of PEPT2 might be regulated by a multiprotein network, which should be clarified by further analysis.

A unique feature of PDZ domain-containing proteins is the presence of multiple PDZ domains in their structure. Since PDZK1 contains four PDZ domains, interaction of each domain with the PEPT2 C-terminus was examined in both yeast two-hybrid and binding assays (Figs. 2 and 3D). The results obtained in both experiments suggest the involvement of PDZ2 and PDZ3 (Figs. 1 and 3C). Additionally, PDZ1 can also interact with PEPT2 C-terminus (Figs. 1 and 3C). However, the deletion of the last four amino acids in PEPT2 C-terminus did not affect the interaction with PDZ1 in both experiments whereas the effect of such deletion was remarkable for the interaction with full-length PDZK1 (Figs. 2A and 3D). Additionally, the growth of yeast cells transfected with PDZ2-3 was remarkable even in the presence of 3-AT where the growth of other cells transfected with either PDZ1-2 or PDZ3-4 was minimal (Fig. 2C). Thus, multiple PDZ domains (PDZ2 and PDZ3) in PDZK1 appear to be involved in the interaction with PEPT2.

In conclusion, PEPT2 interacts with multiple domains in PDZK1, and the signal motif (-TXL) responsible for the in-

teraction resides at the C-terminus of PEPT2. The interaction may be relevant to the functional regulation of PEPT2 by PDZK1. This is the first demonstration of direct protein-protein interaction and functional regulation of oligopeptide transporters and implies the localization of PEPT2 within a protein network constructed with PDZK1 and other transmembrane proteins.

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