INTERACTION WITH PDZK1 IS REQUIRED FOR EXPRESSION OF ORGANIC ANION TRANSPORTING PROTEIN 1A1 (OATP1A1) ON THE HEPATOCYTE SURFACE

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Running title: Interaction with PDZK1 is Required for Oatp1a1 Function

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Although many organic anion transport protein (oatp) family members have PDZ consensus binding sites at their C-termini, the functional significance is unknown. In the present study, we utilized rat oatp1a1 (NM_017111) as a prototypical member of this family to examine the mechanism governing its subcellular trafficking. A peptide corresponding to the C-terminal 16 amino acids of rat oatp1a1 was used to affinity isolate interacting proteins from rat liver cytosol. Protein mass fingerprinting identified PDZK1 as the major interacting protein. This was confirmed by immunoprecipitation of an oatp1a1-PDZK1 complex from cotransfected 293T cells as well as from native rat liver membrane extracts. Oatp1a1 bound predominantly to the first and third PDZ binding domains of PDZK1 while the high density lipoprotein receptor, scavenger receptor B type I (SR-BI) binds to the first domain. Although it is possible that PDZK1 forms a complex with these two integral membrane proteins, this did not occur, suggesting that as yet undescribed factors lead to selectivity in the interaction of these protein ligands with PDZK1. Oatp1a1 protein expression was near normal in PDZK1 knockout mouse liver. However, it was located predominantly in intracellular structures, in contrast to its normal basolateral plasma membrane distribution. Plasma disappearance of the oatp1a1 ligand 35S-sulfobromophthalein was correspondingly delayed in knockout mice. These studies show a critical role for oligomerization of oatp1a1 with PDZK1 for its proper subcellular localization and function. As its ability to transport substances into the cell requires surface expression, this must be considered in any assessment of physiologic function.

A major function of the hepatocyte is the removal of various xenobiotic and endogenous organic anionic compounds from the circulation. Sulfobromophthalein (BSP) is a model organic anion that circulates bound avidly to albumin and is extracted rapidly and efficiently by the hepatocyte (1-3). Establishment of a method to synthesize 35S-BSP of high specific activity (4) facilitated studies to identify its hepatocyte transporter(s). Studies performed in a Xenopus laevis oocyte expression system (5) identified a candidate transporter that was initially termed organic anion transporting polypeptide (oatp). Since this initial description, over 20 additional members of the oatp family have been described (6,7). The original protein was termed oatp1 and subsequently oatp1a1 (Table 1) in a proposal for standardization of nomenclature (7). Studies utilizing antisense knockout of oatp1a1 expression in Xenopus oocytes that had been injected with rat liver mRNA suggested that this protein is responsible for a substantial fraction of organic anion transport by the liver (8), although this remains to be validated by other methods.

The family of organic anion transport proteins (oatp's) is characterized by a high degree of amino acid similarity as well as overlap of transported substrates, although their tissue
distributions are varied (6,7). In addition, they have similar predicted membrane topologies and biochemical characteristics. Although evidence suggests that the oatp's are important in clearance of drugs from the circulation (6,7,9), little is known regarding the mechanism by which they act, their oligomerization state, or mechanisms for subcellular trafficking. Of note is the fact that all of the oatp's that have been examined have distinct plasma membrane distributions, except for the prostaglandin transporters (pgt's) in which intracellular localization appears to predominate (10). Examination of their C-terminal sequences reveals that many of the known members of the oatp family have PDZ consensus binding sites (Table 1). The pgt's are among the group of oatp's that lack a putative PDZ binding domain (11,12).

Generally PDZ consensus binding sites are established by the sequence of the C-terminal 4 amino acids (13-15). Three classes of PDZ consensus binding sites have been described, relating these peptide sequences to the crystal structures of known PDZ domains to which they bind (14). The PDZ consensus sites that are present in the hepatic oatp's are all of Class I, defined by the sequence X-S/T-X-Φ, where X is any amino acid and Φ is a hydrophobic amino acid (14). A relatively large number of PDZ proteins have been described (14,15), although there is as yet no way to predict which if any will bind a particular protein with a PDZ consensus binding site.

In the present study, we utilized rat oatp1a1 (NM_017111) as a prototypical member of the oatp family to examine whether interaction with a PDZ domain-containing protein provides a mechanism governing its subcellular localization. Oatp1a1 is located on the basolateral plasma membrane of the hepatocyte (16), as well as on the apical plasma membranes of the epithelial cells of the choroid plexus and the S3 segment of the renal proximal tubule (16-18). Its terminal 4 amino acids (KTKL) are consistent with a type I PDZ binding motif (14). There is also a mouse homolog of this protein (NM_013797) that is 81% identical to the rat oatp1a1 and the C-terminal 11 amino acids of these two proteins are identical.

MATERIALS AND METHODS

Antibodies and Reagents - Antibody against the N-terminus of oatp1a1 (MEETEKKIATQEGRC) linked to KLH was prepared in rabbits by Covance Research Products Inc. (Denver, PA) as previously described (19). This antibody recognizes oatp1a1 in rat and mouse liver, and was used for immunobLOTS at a 1:1000 dilution. An antibody specific to rat oatp1a1, used in immunobLOTS at a dilution of 1:2500, was raised in rabbits to a KLH-linked peptide corresponding to 13 amino acids near the C-terminus of rat oatp1a1 (aa 646-658) as described previously (16). A rabbit antibody to oatp1a4, used in immunobLOTS at a dilution of 1:10,000, was prepared to a KLH-linked peptide corresponding to the 11 C-terminal amino acids (aa 650-661) of the protein. Rabbit antibodies to SR-BI and PDZK1 were as previously described (20), and were used in immunobLOTS at dilutions of 1:1000. A rabbit polyclonal antibody that recognizes the mouse asialoglycoprotein receptor (ASGPR) was kindly provided by Dr. Richard Stockert for immunofluorescence studies. ECL reagent for Western blot analysis was obtained from PerkinElmer Life Sciences (Shelton, CT). HRP conjugated affinity purified goat anti-rabbit IgG and HRP conjugated affinity purified goat anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA), and were used in immunobLOTS at dilutions of 1:50,000 and 1:10,000, respectively. 293T cells were obtained from Dr. Robert Burk (21). All other reagents were obtained from Sigma unless otherwise noted. All animal procedures were approved by the university committees on animal use.

Preparation of C-terminal Peptide Affinity Gel - The peptides CHGSPQVENDGELKTKL corresponding to the C-terminal 16 amino acids of rat oatp1a1 and CHGSPQVENDGEL, in which the last 4 amino acids were deleted were synthesized in The Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine. An additional cysteine residue was included at the N-terminus to facilitate coupling to Ultralink Idoacetyl Gel (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions.

Isolation of peptide-binding proteins from rat liver cytosol - The liver was surgically removed from a rat under pentobarbital anesthesia and was
immediately infused through the portal vein with 30 ml of ice cold PBS. It was quickly weighed and Dounce homogenized in PBS (10 ml/3 g liver) containing protease inhibitors (Sigma, St. Louis, MO, catalog # P-8340). Following filtration through three layers of cheesecloth, the homogenate was centrifuged at 4°C at 100,000 x g for 1 hr. Supernatant (5 ml) was mixed with 1 ml of the peptide-coupled gel in a column and rotated overnight at 4°C. The gel was then washed successively with 200 ml of ice cold PBS, 10 ml of 0.5 M NaCl, 10 ml of 1 M NaCl and 20 ml of PBS. Proteins remaining bound to the washed gel were eluted with sample buffer and subjected to 10% SDS-PAGE after which they were identified by Coomassie blue or silver staining. 

Mass spectrometry analysis - Coomassie blue-stained bands were excised from SDS-polyacrylamide gels, destained with 0.2 M ammonium bicarbonate in 50% acetonitrile, reduced using 20 mM tris (2-carboxyethyl)-phosphine-HCl, and free cysteine residues were alkylated with 55 mM iodoacetamide. Following digestion for 16 hr at 37°C with 25 ng/µl sequencing grade modified trypsin (Promega), products were cleaned and concentrated using a C18 ZipTip (Millipore), mixed with 0.5 µl of 10 mg/ml 1-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% (v/v) TFA, and applied onto a MALDI plate. Spectra were recorded with a PerSeptive Voyager-DE STR MALDI time-of-flight mass spectrometer operated in the reflection mode. The mass measurement accuracy with internal calibration was better than 100 ppm. The measured peptide masses were used for database searching with ProFound algorithm (ProteoMetrics, NY) and Matrix Science (Mascot). For electrospray ionization MS/MS analysis, an APIQSTAR LC/MS/MS system (Applied Biosystems, Foster City, CA) was used. Fragment ion (tandem) mass spectra were obtained using collision-induced dissociation and analyzed using Matrix Science (Mascot) software.

Immunoprecipitation of rat liver membrane extracts - Antiserum against the N-terminal peptide of oatp1a1 was immunopurified with peptide coupled to Sulfolink agarose (Pierce Biotechnology) according to the manufacturer's instructions. This purified antibody was covalently coupled to immobilized Protein A agarose (Sigma) by incubating for 1 hr at room temperature in 40 mM dimethyl pimelimidate (Pierce) in 0.2 M triethanolamine buffer pH 8.2. A 0.1M Na2CO3 extracted rat liver pellet, highly enriched in oatp1a1 (22), was resuspended in PBS containing 1% Triton X-100 and protease inhibitors (0.1 mM leupeptin, 0.1 mM AEBSF, 0.01 mM Pepstatin A, 1 mM EDTA and 0.01% NaN3). Following centrifugation at 100,000 x g for 1 hr at 4°C, the supernatant was immunoprecipitated with 50 µl of anti-oatp1a1-protein A gel, washed with 1% Triton X-100 in PBS, and 200 µl fractions were eluted with 0.2 M glycine pH 2.3 directly into 1 M Tris-base to neutralize the pH. The eluates were then subjected to Western blot analysis. 

Co-immunoprecipitation of oatp1a1 and PDZK1 following coexpression in 293T cells - Oatp1a1 cDNA was excised from pSPORT-oatp1a1 using the KpnI and NotI multicloning restriction enzyme sites (23) and cloned into pCDNA3.1(+) (Invitrogen Life Tech. Carlsbad, CA). PDZK1 cDNA was cloned into pFLAG-CMV-5c (Sigma) following PCR-amplification from a pCDNA3.1/hygro-PDZK1 plasmid (20), resulting in a plasmid encoding PDZK1 with FLAG at its N-terminus. Transient co-transfection of 293T cells with pCDNA3.1-oatp1a1 and pFLAG-CMV/PDZK1 was performed using PolyFect transfection reagent (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Cells were harvested two days after transfection, washed with PBS, and incubated for 30-60 min on ice with PBS containing 1% CHAPS and protease inhibitors (Sigma catalog # P-8340). The lysate was centrifuged at 100,000 x g for 30 min at 4°C and the supernatant was incubated overnight at 4°C with oatp1a1-protein A affinity gel or anti-FLAG M2 affinity gel (Sigma) (25 µl gel/4 x 106 cells). Each gel was washed with 1% CHAPS in PBS, incubated with SDS-PAGE sample buffer, centrifuged, and the supernatant was subjected to Western blot analysis. A control study was also performed in which oatp1a4 and FLAG-PDZK1 were cotransfected into 293T cells which were then subjected to FLAG immunoprecipitation as described above. For these studies, oatp1a4 cDNA was excised from a pCR2.1-oatp1a4 plasmid that was provided by Dr. Richard Kim (24). The oatp1a4 insert was excised using KpnI and XhoI multicloning restriction enzyme sites,
and was cloned into the pCDNA3.1/Zeo(-) plasmid.

Identification of oatp1a1-interacting PDZK1 domains - As previously described, PDZK1 has 4 independent PDZ binding domains (25,26). pGEX6p-1 GST expression plasmids (Amersham Pharmacia Biotech, Piscataway, NJ) containing cDNA's encoding each of these domains were prepared, using the pCDNA3.1/hygro-PDZK1 plasmid as PCR template. For amplification of intact PDZK1 a sense primer (5’ ACTTAAGGATCCACAGAATGGGCTTCAC CTTCAAC 3’) and antisense primer (5’ ACGGGCCTCTCAGACTCAG 3’) encoding BamHI and XhoI restriction sites, respectively, were used. For the first domain (aa 1-110) sense (5’ CGCGGATCCATGGCCTCCACCTTTCAAC CCAGAGAGTGT 3’) and antisense (5’ CCGCTCGAGCTACCTCTGGCTTTGATCCAG CTCTTTCAA 3’) primers encoding BamHI and XhoI restriction sites, respectively, were used and for domains 2-4 (aa 113-235, aa 221-343 or aa 356-519, respectively), the sense and antisense primers encoded EcoRI and XhoI restriction sites, respectively. The primers used for domain 2 are 5’ CCGGAATTCGCTCTGAATGATAAGAAACCGGCCCTGGG 3’ (sense) and 5’ CCGCTCGAGTTACAAACTGGCTGTCTCCCTTGAATTGTGT 3’ (antisense). The primers used for domain 3 are 5’ CCGCAATTCAGTGAACAGAAGACACAATTCAAGAGGGAG 3’ (sense) and 5’ CCGCTCGAGTTATTCTTGACTTTGGCAGTAAGAAGTGGAGA 3’ (antisense). The primers used for domain 4 are 5’ CCGGAATTCATCCCTGCTCCTCTGGAGGCCACAGGCTCA 3’ (sense) and 5’ CCGCTCGAGTCACATCTCCGTGTCTTCAGAGTTAGACGAAGA 3’ (antisense). The GST fusion proteins were expressed in E. Coli (DH5α, Invitrogen) and affinity purified using GSH-agarose gel (Sigma). Briefly, the bacteria were grown overnight in 5 ml of LB/ampicillin medium at 37°C. The culture was then diluted into 50 ml of LB/ampicillin medium and grown at 37°C until the OD_{600} was 0.6-1 absorption units. Expression of fusion proteins was induced by addition of 0.1 mM IPTG (Sigma) followed by an additional 4 hr incubation. The bacteria were harvested, resuspended in 1% Triton X-100 in PBS (5 ml/50 ml bacterial culture) containing protease inhibitor (Sigma catalog # P-8340), and sonicated on ice. The bacterial lysate was centrifuged at 12,000 rpm (Sorvall RC-5B centrifuge) for 15 min at 4°C and the supernatant was rotated with GSH-agarose gel overnight at 4°C. The gel was washed several times with 1% Triton X-100 in PBS, resuspended in extracted rat liver membrane containing protease inhibitors as prepared above (5 mg membrane/15 μl gel) and rotated overnight at 4°C. The gel was washed with 1% Triton X-100 in PBS 5 times before addition of SDS-PAGE sample buffer for Western blot analysis.

Generation of PDZK1 gene targeted mice - Mice in which exon1 and part of intron1 of the PDZK1 allele were replaced by the Neo cassette were prepared and bred in the Columbia University Transgenic Facility as described previously (27).

Immunofluorescence localization of PDZK1 in liver - Male wild type or PDZK1 knockout mice were anesthetized with ether, and the livers were removed and fixed by immersion for 3 hr at 4º C with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, containing 7.5% sucrose and frozen sections (~30 µm thick) were prepared (16). Sections were exposed overnight at 4°C to anti-oatp1a1, anti-ASGPR, or anti-PDZK1 diluted 1:100 in PBS and, after rinsing in PBS, were exposed overnight at 4°C to a 1:400 dilution of a Cy3-labeled donkey antibody to rabbit IgG (Jackson ImmunoResearch). Controls included examination of sections for autofluorescence after exposure to nonspecific primary antiserum. Wide field, immunofluorescence images were captured with a 60X Olympus objective (1.4 NA) on an Olympus IX71 microscope. Rhodamine fluorescence was excited with a DG-4 (Sutter Instruments) xenon light source and digital images were recorded on a Photometrics CoolSnap HQ CCD camera controlled by Metamorph imaging software (Universal Imaging Corp). For deconvolution, liver was optically sectioned every 0.25 µm using an MS-2000 automated piezoelectric X, Y, Z stage (Applied Scientific Instruments) for a total of 72 optical sections. Image stacks were deconvolved using the Metamorph 'measured deconvolution' algorithm. For this, sub-optical resolution fluorescent beads (PS-Spec, Molecular Probes) were sectioned in the Z dimension under identical conditions as the liver. Bead stacks were assembled to generate a
measured 'point spread function' and this point spread function was used to optically deconvolve the stacks of liver fluorescence images.

$^{35}$S-BSP plasma disappearance - $^{35}$S-BSP (1000 µCi/µmole) was synthesized as previously described (4). Male wild type and PDZK1 knockout mice were anesthetized with ketamine and injected retroorbitally with approximately 140,000 cpm of $^{35}$S-BSP in 0.1 ml PBS. Timed blood samples (~ 40 µl) were obtained from the contralateral retroorbital sinus in heparinized capillary tubes, which were then centrifuged and radioactivity in an aliquot of plasma was quantified. Plasma disappearance of radioactivity as a fraction of the injected dose was fit by a non-linear least squares algorithm (SigmaPlot v 6.1, SPSS, Inc., Chicago, IL) to the single exponential equation $C(t) = ae^{-bt}$, where $C(t)$ is the fraction of injected $^{35}$S-BSP/ml plasma at time t, a is the fraction of injected $^{35}$S-BSP/ml plasma at time 0, and b is the fractional disappearance rate in sec$^{-1}$. The volume of distribution of $^{35}$S-BSP was calculated as 1/a, and the serum half-life of $^{35}$S-BSP ($t_{1/2}$) was calculated as ln(2)/b.

RESULTS

Isolation and identification of rat liver cytosolic proteins that bind to the C-terminal tail of oatp1a1 - For these experiments, a peptide corresponding to the C-terminal 16 amino acids of rat oatp1a1 was covalently coupled to agarose gel. This peptide minus the last 4 amino acids that comprise the PDZ binding consensus domain was also coupled to agarose gel and used as a control. The columns were washed extensively with PBS as well as 1M NaCl. Proteins bound to the washed gel were eluted with SDS-PAGE sample buffer and detected following SDS-PAGE by silver stain or staining with Coomassie blue. A representative silver stained SDS-PAGE gel is seen in Figure 1a. A major protein band of approximately 70 kDa was detected only in the material that was bound to the intact peptide (lane 1) and not to the peptide lacking the terminal 4 amino acids (lane 2). This band was visualized following Coomassie blue staining of replicate gels and was excised. Following reduction and alkylation, the gel slice was incubated overnight with trypsin. The resulting tryptic peptides were identified by matrix assisted laser desorption/ionization (MALDI) mass spectrometry. A representative MALDI mass spectrum is shown in Figure 1b. Database analysis revealed a high correspondence of the observed peptide masses to those that would be obtained by tryptic digestion of rat PDZK1. These 64 possible tryptic peptides are indicated in Figure 1c by alternating shading that has been applied to the PDZK1 sequence. Following analysis of multiple MALDI spectra, fifty peptides corresponding to 66% of the protein sequence of PDZK1 were identified as indicated by the solid underlines in Figure 1c. Identification as PDZK1 was confirmed by tandem MS/MS analysis. A representative MS/MS spectrum is shown in Figure 1d, in which the sequence corresponding to the fifth PDZK1 tryptic peptide was identified. Masses in this figure are annotated using standard nomenclature as described (28). Utilizing tandem MS/MS, the sequences of 27 peptides corresponding to 54% of the PDZK1 sequence were identified, and are indicated by the broken underlines in Figure 1c. Together, the two methods identified 93% of the PDZK1 sequence (Figure 1c). In addition, identification of this protein as PDZK1 was confirmed by immunoblot using a peptide-specific antibody (data not shown).

Interaction of oatp1a1 and PDZK1 in cells and rat liver - These studies showed that the C-terminal 4 amino acids of oatp1a1 are necessary for interaction with PDZK1. However, these results are not predictive as to whether this interaction between oatp1a1 and PDZK1 actually occurs in vivo. Although several proteins have been shown to bind to PDZK1 under in vitro conditions, the functional significance of this interaction has not always been clear (29,30). To assess whether the interaction found with the C-terminal peptide occurs with full-length proteins expressed in cells, 293T cells were transfected with expression plasmids encoding oatp1a1 and FLAG-PDZK1. Immunoprecipitation was performed with FLAG antibody or an antibody raised to the N-terminus of oatp1a1 that would not be expected to interfere with interaction of the C-terminus with PDZK1. Western blot analysis of the FLAG immunoprecipitate with anti-oatp1a1 revealed the presence of oatp1a1 (Figure 2a, left panel) and Western blot analysis of the oatp1a1 immunoprecipitate revealed the presence of FLAG-PDZK1 (Figure 2a, right panel).
was no product detected in the immunoprecipitate following cotransfection of either expression plasmid with the alternate empty plasmid (Figure 2a, lanes 2 and 3). As a control, coexpression of oatp1a4 and FLAG-PDZK1 in 293T cells revealed that the FLAG-PDZK1 immunoprecipitate did not contain detectable oatp1a4 (Figure 2b). Rat oatp1a4, formerly known as oatp2, is a member of the oatp family with an amino acid sequence that is approximately 76% identical to that of oatp1a1. As seen in Table 1, although it is distributed on the basolateral plasma membrane of the hepatocyte, it lacks a PDZ consensus sequence at its C-terminus. These studies of oatp1a1-FLAG-PDZK1 interaction were performed in cells in which synthesis of these proteins was maximized. They do not prove that such an interaction actually occurs in hepatocytes, where protein concentrations may be lower and other proteins may be competing for binding to PDZK1. Thus, although these experiments indicate that full-length PDZK1 can interact with full-length oatp1a1, it is important to determine whether this interaction actually occurs in the liver. Consequently, a series of experiments was performed in which a rat liver membrane detergent extract was subjected to immunoprecipitation with antibody to the N-terminus of oatp1a1. The immunoprecipitates were subjected to SDS-PAGE following which Western blot analysis was performed. As seen in the left two panels of Figure 2c, antibody to oatp1a1 immunoprecipitated oatp1a1 as well as PDZK1, confirming that they are bound to each other in liver. Perhaps the best characterized ligand partner for PDZK1 is the high density lipoprotein (HDL) receptor, scavenger receptor B type I (SR-BI). This protein is present on the basolateral surface of hepatocytes, where it selectively extracts lipids from HDL particles (20,31). Previous studies have shown that SR-BI and PDZK1 coimmunoprecipitate from rat liver (25). Interestingly, as seen in the fourth panel of Figure 2c, there was no SR-BI found in the oatp1a1 immunoprecipitate from rat liver. Rat oatp1a4, as noted above, lacks a PDZ consensus sequence at its C-terminus. We hypothesized that it might sort to the cell surface as a complex with oatp1a1, but as seen in the third panel of Figure 2c, it is not present in the oatp1a1 immunoprecipitate from rat liver. These results indicate that there is specific interaction of oatp1a1 and PDZK1 in the liver.

PDZK1 has 4 independent PDZ domains (25,26). SR-BI binds to the first PDZ domain of PDZK1 (25). Experiments were designed to determine the domain(s) to which oatp1a1 binds. Plasmids encoding PDZK1 binding domains 1 (aa 1-110), 2 (aa 113-235), 3 (aa 221-343), or 4 (aa 356-519) as GST fusion proteins were constructed. The respective proteins were expressed in E. Coli and bound to GSH-agarose gels. Each gel was incubated with a Triton X-100 rat liver membrane extract. Gels were then washed extensively and bound proteins were eluted into SDS-PAGE sample buffer and subjected to Western blot analysis using antibody to oatp1a1. As seen in Figure 2d, oatp1a1 bound predominantly to the first and third domains of PDZK1.

Oatp1a1 expression and function in PDZK1 knockout mice - Although the preceding studies suggest a strong interaction of oatp1a1 with PDZK1 in the liver, they do not prove that this interaction is physiologically relevant. To examine this issue, studies were performed in mice in which expression of PDZK1 was genetically disrupted. The mouse homolog of rat oatp1a1 has the identical PDZ recognition motif at the C-terminus, and is recognized by the antibody to the N-terminus of oatp1a1 that was used in the immunoprecipititation studies. Previous studies in transgenic mice revealed that interaction with PDZK1 was essential for targeting of SR-BI to the hepatocyte plasma membrane (20). In concordance with these observations, SR-BI expression and function in livers from PDZK1 knockout mice were found to be markedly reduced (27,32). An as yet unexplained post-transcriptional process is responsible for this reduction in protein expression, as levels of mRNA encoding SR-BI were normal in these mice (32). We confirmed by Western blot that the strain of PDZK1 mice that were used in the present study had no expression of PDZK1 protein as compared to wild type mice (Figure 3a, top panel). Similar to the earlier studies noted above that were performed in another strain of PDZK1 knockout mice (32), this was accompanied by a substantial reduction in expression of SR-BI (Figure 3a, middle panel). In contrast, total expression of oatp1a1 in liver homogenate was similar to wild type when determined in PDZK1
knockout mice (Figure 3a, bottom panel). However, when subcellular distribution of oatp1a1 was examined in liver from knockout mice, it was found to be predominantly in intracellular structures, in contrast to the typical basolateral distribution of oatp1a1 (16) in wild type mouse liver (Figure 3b, top panels). Importantly, the absence of PDZK1 did not cause a general disturbance in membrane behavior since the basolateral distribution of the asialoglycoprotein receptor (ASGPR) in livers from normal and knockout mice were indistinguishable (Figure 3b, middle panels). As expected, PDZK1 was undetectable in the knockout and had a primarily basolateral distribution in the wild type liver (Figure 3b, bottom panels). Much of the cytosolic pool of PDZK1 likely washed out during the preparation and permeabilization of the tissue.

The functional significance of this subcellular redistribution of hepatocyte oatp1a1 was determined by quantifying plasma disappearance of 35S-BSP. BSP is a well characterized substrate for oatp1a1-mediated cellular uptake (8,9,22), although several other hepatocyte plasma membrane proteins also have the ability to mediate its uptake (9). This probably accounts for the fact that plasma disappearance of BSP was relatively rapid in both wild type and PDZK1 knockout mice (Table 2). Analysis of these data by non-linear least squares regression revealed that the plasma volume of distribution was identical in wild type and knockout mice (Table 2). However, the fractional uptake rate of BSP was reduced by approximately 25% (p<0.05) and the corresponding plasma half-life was increased by the same proportion (p<0.05) in the PDZK1 knockout as compared to wild type mice (Table 2).

DISCUSSION

The present study establishes that oatp1a1 binds to PDZK1 in vitro and in vivo. PDZK1 is a 70 kDa protein with 4 independent PDZ domains that has been shown to be present in a number of tissues including liver and kidney (20,25,26,32,33). Although several proteins have been shown to bind to PDZK1 under in vitro conditions, the functional significance of this interaction has not always been clear (30). Perhaps the best characterized ligand partner for PDZK1 is the high density lipoprotein receptor, SRBI. SRBI and PDZK1 coimmunoprecipitate from rat liver, and studies in transgenic mice reveal that this interaction is essential for targeting of SRBI to the hepatocyte plasma membrane (20). In concordance with these observations, there is markedly reduced SRBI expression and function in livers from PDZK1 knockout mice (27,32). The present study indicates that interaction with PDZK1 is also essential for hepatocyte plasma membrane expression of oatp1a1. SRBI binds to the first PDZ domain of PDZK1 (25), while oatp1a1 binds predominantly to the first and third domains (Figure 2d). Although it is thus possible that PDZK1 could form a complex with these two membrane proteins, we have found no evidence for this (Figure 2c), suggesting that as yet undescribed factors lead to selectivity in the interaction of these protein ligands with PDZK1.

Although mouse oatp1a4 has been described as the homolog of rat oatp1a4 (34), the mouse protein has a C-terminal PDZ binding consensus site (KTKL) while the rat protein does not (Table 1). These proteins are 89% identical although the C-terminus of the mouse protein is 8 amino acids longer than that of the rat protein. Despite the lack of a PDZ binding domain, the rat protein localizes to the basolateral plasma membrane of the hepatocyte (35). Although it is possible that oatp1a4 traffics to the plasma membrane as a complex with oatp1a1, we found no evidence for their binding to each other as determined by failure to recover oatp1a4 following immunoprecipitation of oatp1a1 from rat liver lysate (Figure 2c). As shown in the present study, expression of oatp1a1 at the plasma membrane requires its interaction with PDZK1 and apparently cannot utilize the as yet undescribed PDZ-independent mechanism that is utilized by oatp1a4.

As noted above, the prostaglandin transporters (pgt's), members of the oatp family, also lack PDZ consensus binding sites. Immunolocalization of pgt in various rat tissues reveals a predominant intracellular distribution (10), while oatp1a1 is predominantly on the plasma membrane of hepatocytes, renal tubules, and choroid plexus epithelial cells (16-18). It is interesting that in the choroid plexus, oatp1a1 is distributed in large intracellular vesicular structures during the initial 8-10 wks of development before assuming the adult apical...
plasma membrane phenotype (18). Whether this is a result of altered expression of PDZK1 during development is not known at the present time.

The present study shows that interaction of oatp1a1 with PDZK1 is required for its expression on the hepatocyte surface. As its ability to transport substances into the cell requires surface expression, this must be considered in any assessment of its physiologic function. Most studies of oatp1a1 function have been performed in transfected cells, lacking PDZK1, in which the transporter is overexpressed. Whether the lack of a PDZK1 binding scaffold will have an affect on oatp1a-mediated transport function is an important subject that will need to be addressed in future studies.

REFERENCES

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FIGURE LEGENDS

Figure 1. Identification of rat liver cytosolic proteins that bind to the C-terminal tail of oatp1a1. (a) The peptides CHGSPQVENDGELKTKL corresponding to the C-terminal 16 amino acids of rat oatp1a1 (lane 1) and CHGSPQVENDGEL, in which the last 4 amino acids were deleted (lane 2) were coupled to iodoacetyl agarose gel, incubated with rat liver cytosol and then washed extensively with PBS and 1 M NaCl before proteins were eluted into sample buffer and resolved on 10% SDS-PAGE. A representative silver-stained gel is shown. The arrowhead indicates the major band that was further analyzed by mass spectrometry. (b) A Coomassie blue-stained band, corresponding to that indicated by the arrowhead in panel a, was excised from a replicate SDS-polyacrylamide gel and subjected to in-gel digest with trypsin. A representative MALDI-TOF MS mass fingerprint is shown. Peptides corresponding to those theoretically obtained from tryptic digestion of rat PDZK1 (panel c) are indicated. (c) The 64 peptides (T1-T64) that can theoretically be obtained following tryptic digestion of PDZK1 are indicated by alternating shading that has been applied to the PDZK1 sequence. Database analysis identified multiple peptides that corresponded to the theoretical tryptic peptides of rat PDZK1. The solid underlines show peptides identified by MALDI-TOF MS (66% of the protein sequence) and the broken underlines show peptides identified by ESI-Q-TOF MS (54% of the protein sequence). Together, the two methods identified 93% of the rat PDZK1 sequence. (d) A representative CID MS/MS product ion spectrum from Q-TOF MS is shown. This identifies [M +2H]2+ fragmentation of the T5 tryptic peptide of rat PDZK1.
Figure 2. Interaction of oatp1a1 and PDZK1 after coexpression and in native liver. (a) Oatp1a1 and FLAG-PDZK1 were co-transfected into 293T cells. A lysate was prepared in 1% CHAPS and immunoprecipitations were performed using anti-oatp1a1 (right panel) or anti-FLAG (left panel) coupled to agarose beads. Western blots, performed with the opposite antibodies, showed specific coimmunoprecipitation of the two proteins. Cotransfections were: lane 1: pCDNA3.1-oatp1a1/FLAG-PDZK1; lane 2: pCDNA3.1-oatp1a1/FLAG-vector; lane 3: pCDNA3.1-vector/FLAG-PDZK1. (b) Oatp1a4 and FLAG-PDZK1 were co-transfected into 293T cells. A lysate was prepared in 1% CHAPS and immunoprecipitations were performed using anti-FLAG coupled to agarose beads. Western blot of 50 µl of 1ml total lysate (Lys) and 50µl of 75 µl immunoprecipitate eluate (IP) for oatp1a4 (arrowhead, left panel) revealed that it was not immunoprecipitated with FLAG-PDZK1 which was present in the immunoprecipitate as shown in the Western blot with FLAG antibody in the right panel. (c) Na₂CO₃ extracted rat liver membranes were prepared and solubilized in 1% Triton X-100. Following immunoprecipitation with anti-oatp1a1 covalently coupled to agarose, bound proteins were released by 0.2 M glycine pH 2.3 and collected directly into 1 M Tris-base to neutralize the pH. Immunoblots of 50 µl of 1ml total lysate (Lys) and 50µl of 75 µl immunoprecipitate eluate (IP) were then performed using antibodies to oatp1a1 (C-terminal), PDZK1, oatp1a4, or SR-BI as indicated. (d) The 4 PDZ domains of PDZK1 were expressed as GST fusion proteins and bound to GSH-agarose. Each gel was incubated with a 1% Triton X-100 rat liver membrane extract and following extensive washing, bound proteins were eluted into SDS-PAGE sample buffer and analyzed by Western blot with oatp1a1 antibody. PDZK1 domains: 1: aa 1-110; 2: aa 113-235; 3: aa 221-343; 4: aa 356-519. Relative quantitation of the oatp1a1 bands by densitometry is shown at the bottom of this representative gel.

Figure 3. Protein expression in livers from wild type and PDZK1 (-/-) mice. (a) Livers were removed from two mice in each group and Dounce homogenized in 1 mM NaHCO₃. 20 µg of protein was applied to each lane of a 10% SDS-PAGE and analyzed by Western blot using antibodies to PDZK1 (upper panel), SR-BI (middle panel), or oatp1a1 (lower panel). (b) Immunofluorescence for oatp1a1, the asialoglycoprotein receptor (ASGPR), and PDZK1 was performed on sections of wild type or PDZK1 (-/-) livers and analyzed following computer deconvolution. Single representative images from the deconvolved stacks are presented. In the liver from PDZK1 (-/-) mice, oatp1a1 is expressed only intracellularly in contrast to the basolateral distribution in liver from PDZK1 (+/+) mice. Scale bar = 20 µm.
Table 1  Members of the oatp family that are found in rat, mouse, or human liver

<table>
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<tr>
<th>Species</th>
<th>Original Name</th>
<th>New Name</th>
<th>NCBI Accession Number</th>
<th>C-Terminal Sequence</th>
<th>Potential PDZ Consensus</th>
<th>Plasma Membrane</th>
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<tr>
<td>Rat</td>
<td>Oatp1</td>
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<td>NM_017111</td>
<td>KTKL</td>
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<td>Oatp1a4</td>
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<td>LQEL</td>
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<td>DSRV</td>
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ND: Not determined
Table 2: Kinetic parameters of plasma disappearance of $^{35}$S-BSP in wild type (WT) and PDZK1 (-/-) mice.

<table>
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<th></th>
<th>n</th>
<th>a (ml$^{-1}$)</th>
<th>b (sec$^{-1}$)</th>
<th>$r^2$</th>
<th>VD (ml)</th>
<th>$t_{1/2}$ (sec)</th>
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<tr>
<td>WT</td>
<td>5</td>
<td>0.71 ± 0.07</td>
<td>0.013 ± 0.003</td>
<td>0.84 ± 0.11</td>
<td>1.41 ± 0.14</td>
<td>55.5 ± 14.6</td>
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<tr>
<td>PDZK1 (-/-)</td>
<td>4</td>
<td>0.83 ± 0.42</td>
<td>0.0098 ± 0.0009*</td>
<td>0.82 ± 0.29</td>
<td>1.39 ± 0.59</td>
<td>70.8 ± 5.97*</td>
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Data were fit by non-linear least squares regression to the equation $C(t) = ae^{-bt}$, where $C(t)$ is the fraction of injected $^{35}$S-BSP/ml plasma at time $t$, $a$ is the fraction of injected $^{35}$S-BSP/ml plasma at time 0, and $b$ is the fractional disappearance rate in sec$^{-1}$. $r^2$ is the correlation coefficient of the fit. The volume of distribution of $^{35}$S-BSP (VD) is calculated as $1/a$, and the serum half-life of $^{35}$S-BSP ($t_{1/2}$) is calculated as $\ln(2)/b$. *p< 0.05
Figure 2

(a) Western blot of oatp1a1 with FLAG and Western markers.
(b) FLAG-IP of oatp1a4 with Lys and Western markers.
(c) Lys and IP of oatp1a1, PDZK1, oatp1a4, and SR-BI.
(d) Densitometric quantitation of PDZK1 Domain.
Figure 3

(a) Western blot analysis of PDZK1, SR-BI, and oatp1a1.

(b) Immunofluorescence images of OATP1A1 and ASGPR in PDZK1 (+/+) and PDZK1 (-/-) conditions.
Interaction with PDZK1 is required for expression of organic anion transporting protein 1A1 (OATP1A1) on the hepatocyte surface

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