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Interaction of Human OATP1B1 with PDZK1 Is Required for Its Trafficking to the Hepatocyte Plasma Membrane

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ABSTRACT

Uptake of xenobiotics by hepatocytes is mediated by specific proteins, including organic anion transporting polypeptides (OATPs), residing on the basolateral (sinusoidal) plasma membrane. Many of the OATPs have PDZ consensus binding sites, determined by their C-terminal 4 amino acids, while others do not. Mouse and rat OATP1A1 are associated with PDZK1, which is necessary for their trafficking to the plasma membrane. humanOATP1B1 (hOATP1B1) is a major drug transporter in human liver. Although localized to the plasma membrane, it was thought to lack a PDZ consensus motif, suggesting that the trafficking paradigm for murine OATPs is not applicable to human liver. The aim of the present study was to determine whether hOATP1B1 is a ligand for hPDZK1. hOATP1B1 immunoprecipitates with hPDZK1 following co-expression in 293T cells as well as in normal human liver. Co-expression with each of the 4 PDZ domains revealed interaction with domain 1 only. A truncated version of hOATP1B1 that lacks its terminal 4 amino acid PDZ binding motif as well as hOATP1B3, which does not contain a PDZ binding consensus motif, failed to interact with hPDZK1. Immunofluorescence microscopy of hOATP1B1 in stably transfected HeLa cells that endogenously express hPDZK1 showed that it distributes predominantly along the plasma membrane whereas hOATP1B1 lacking its terminal 4 amino acids distributes primarily intracellularly with little plasma membrane localization. Similar to findings in rats and mice, human OATP1B1 is a ligand for PDZK1 and requires interaction with PDZK1 for optimal trafficking to the hepatocyte plasma membrane.

SIGNIFICANCE

Previous studies suggested that OATP1B1, a major xenobiotic transporter in human liver, does not have a PDZ binding consensus motif and does not follow the paradigm for subcellular trafficking and function that was established for OATP1A1 in murine liver. We now demonstrated that OATP1B1 but not OATP1B3 has a PDZ binding consensus motif that mediates binding to PDZK1 and is required for its trafficking to the plasma membrane. Such interaction could be an important previously unrecognized modulator of transport function.

Introduction

The hepatocyte plays an important role in removal of various compounds from the circulation. For the most part, this transport activity is mediated by specific proteins that are localized on its basolateral (sinusoidal) plasma membrane. Many of these compounds have molecular weights of less than 1000 kDa and are negatively charged at physiologic pH. They have been broadly classified as organic anions and can be removed efficiently from the circulation with single-pass extraction as high as 50% or more (Wolkoff, 2014). Previous studies performed in rat liver identified a 75 kDa glycoprotein, initially named organic anion transporting polypeptide (OATP), whose expression in various model systems was sufficient to mediate transport of a number of substrates, including the organic anion sulfobromophthalein (Jacquemin et al., 1994; Shi et al., 1995). OATP, now known as OATP1A1, was subsequently found to be the first member of a new family of transport proteins in the solute carrier organic anion transporter (SLCO) family, which have overlapping substrate specificities and tissue distributions (Hagenbuch and Stieger, 2013; Wolkoff, 2018; Schulte and Ho, 2019). The OATPs are 12 transmembrane domain glycoproteins that are largely localized to the plasma membrane (Wang et al., 2008). Many of the OATPs have PDZ consensus binding sites at their C-termini (Anwer and Wolkoff, 2020). These sites are determined by their C-terminal 4 amino acids and can mediate interaction with specific PDZ proteins, forming multiprotein complexes (Amacher et al., 2020). In particular, we found that mouse and rat OATP1A1 are associated with PDZK1 and that this association is necessary for trafficking to the hepatocyte basolateral plasma membrane (Wang et al., 2005, 2014; Choi et al., 2011). In PDZK1 knockout mice, mOATP1A1 expression on the hepatocyte surface was markedly reduced and the transporter was seen to accumulate in intracellular vesicles resembling endocytic vesicles (Wang et al., 2014). In wild-type mouse liver, OATP1A1-containing endocytic vesicles were also associated with PDZK1. PDZK1 association was

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ABBREVIATIONS: AU, arbitrary units; FT, flow-through; OATP, organic anion transporting polypeptide; PCR, polymerase chain reaction; PDZ, postsynaptic density protein, drosophila disc large tumor suppressor, zonula occludens-1 protein; PDZK1, PDZ domain containing 1; sfGFP, superfolder green fluorescent protein; SLCO, solute carrier organic anion transporter.

required for binding and movement of these vesicles along microtubules, resulting in trafficking between the cell surface and intracellular destinations (Wang et al., 2014). This trafficking was mediated by selective recruitment by PDZK1-containing vesicles of kinesin-1, a plus end directed motor molecule that traffics its cargo along microtubules toward the cell surface (Wang et al., 2014). Vesicles prepared from PDZK1 knockout mice were largely associated with dynein, a minus end directed motor molecule that traffics its cargo away from the cell surface. These studies have provided a paradigm for regulation of OATP trafficking. However, although many members of the OATP family possess PDZ consensus binding motifs at their C-termini, many do not. For example, rat OATP1A4 lacks a PDZ binding motif yet still is localized to the hepatocyte basolateral plasma membrane (Wang et al., 2019). Recent studies showed it traffics by interacting with rOATP1A1, functionally hitchhiking in a complex through the cell (Wang et al., 2019). Several OATPs have been described in human liver. Of these, hOATP1B1 is thought to play a major role in drug transport and has also been shown to interact with hOATP1B3 (Zhang et al., 2017). Although hOATP1B1 has a clear plasma membrane localization, it has been thought to lack a PDZ binding consensus motif (Chun et al., 2017). However, analysis of hOATP1B1 using a newer algorithm (http://pow.baderlab.org) has suggested that its C-terminal amino acid sequence (ETHC) is a ligand for hPDZK1 (Hui et al., 2013; Wang et al., 2019). Similar in silico analysis of hOATP1B3 failed to show presence of a PDZ binding consensus motif. The present study was designed to test these predictions.

Materials and Methods

Plasmids

Preparation of pMEP4-hOATP1B1 and pMEP4-hOATP1B1$^{\Delta ETHC}$ Expression Plasmids. Human OATP1B1 cDNA cloned in the plasmid pEF6 was a gift from Dr. Richard Kim (University of Western Ontario, Canada) (Cvetkovic et al., 1999; Taniguchi et al., 2020). The hOATP1B1 cDNA was recloned into pMEP4, a mammalian expression vector in which expression is regulated by the zinc-inducible metallothionein IIa promoter (Shi et al., 1995). In brief, the hOATP1B1-pEF6 plasmid was digested with KpnI and NotI, and the resulting hOATP1B1 was ligated into pMEP4 that had been predigested with KpnI and NotI (Wang et al., 2003). A construct was also prepared encoding a truncated hOATP1B1 (hOATP1B1$^{\Delta ETHC}$) lacking the C-terminal 4 amino acids (ETHC) that comprise its PDZ consensus domain. In brief, pMEP4-hOATP1B1 cDNA was used as a template for polymerase chain reaction (PCR) amplification using 5′-CGGGGTACCATGGACCAAAATCAACATTTGAATAAAACAGCAGAG-3′ as the sense primer and 5′-CCGCTCGAGTTAACTATCTGCCCCAGCAGAAGG GACAAAATGTTT-3′ as the antisense primer for hOATP1B1$^{\Delta ETHC}$. These sense and antisense primers also encoded KpnI and XhoI restriction sites, respectively. The PCR-amplified product encoding hOATP1B1$^{\Delta ETHC}$ was digested with KpnI and XhoI and ligated into the pMEP4 plasmid. Its sequence was verified by automated sequencing in the DNA Sequencing Facility of the Albert Einstein College of Medicine.

Preparation of sfGFP-hOATP1B1 and sfGFP-hOATP1B1$^{\Delta ETHC}$ Expression Plasmids. A superfolder GFP (sfGFP) encoding plasmid (Pedelacq et al., 2006) was provided by the Imaging and Cell Structure Core of the Marion Bessin Liver Research Center at the Albert Einstein College of Medicine. pMEP4-hOATP1B1 and pMEP4-hOATP1B1$^{\Delta ETHC}$ served as templates for preparation of the respective transporter cDNAs using 5′-CCGCTCGAGGATCCA TGGACCAAAATCAACATTTGAATAAAACA-3′ as the sense primer and 5′-CGGGGTACCTTAACAATGTGTTTCACTATCTGCCCCAGCA-3′ or 5′-GGTG GATCCCGGGCCCGCGGTACCTTAACAATGTGTTTAACTATCTGCCC CAGC AGAAGGGAC-3′ as antisense primers for the wild-type and truncated cDNAs, respectively. These PCR products retained the XhoI and KpnI restriction sites, which were used for ligation into the sfGFP plasmid.

Preparation of sfGFP-hOATP1B3 and sfGFP-hOATP1B3$^{\Delta AAAN}$ Expression Plasmids. An expression plasmid encoding OATP1B3 (pCMV6-OATP1B3) was a gift from Dr. I. David Goldman of the Albert Einstein College of Medicine. cDNA for insertion into the sfGFP plasmid was prepared from this plasmid by PCR using 5′CCG CTC GAG GAA TGG ACC AAC ATC AAC ATT TGA ATA AAA CA 3′ as the sense primer and 5′ T TCC GCG GCC GCT ATG GCC GAC GTC GAC TTA GTT GGC AGC AGC ATT GTC TTG CAT GTC 3′ as the antisense primer. The PCR products retained the XhoI and SalI restriction sites, which were used for ligation into the sfGFP plasmid.

Preparation of sfGFP-hOATP1B3$^{\Delta AAAN}$ was by mutagenesis of wild-type sfGFP-hOATP1B3 using 5′ GAC ATG CAA GAC AAT ACT GCT GCC AAC TAAGTC GAC 3′ as a sense primer and 5′ GTC GAC TTA GTT GGC AGC AGT ATT GTC TTG CAT GTC 3′ as an antisense primer. The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's directions. The mutagenized plasmid DNA was extracted with the Promega Plasmid mini prep kit (Promega, WI), and mutations were verified by automated sequencing in the DNA Sequencing Facility of the Albert Einstein College of Medicine.

Preparation of Expression Plasmids Encoding FLAG-hPDZK1 and Individual PDZ Domains 1 to 4. N-flag-pEZ-hPDZK1 plasmid (EX-Q0230-M11) expressing full-length hPDZK1 with FLAG at the N-terminus was obtained from GeneCopoeia, (Rockville, MD). Similar to murine PDZK1, hPDZK1 has four independent PDZ consensus binding domains (Anzai et al., 2004). p3xFlag-CMV-7.1 expression plasmids (Sigma) containing cDNAs encoding each of these domains were prepared using the N-flag-pEZ-hPDZK1 plasmid as PCR template. Primers were prepared to encode NotI and EcoRV restriction sites for each of the four domains (https://www.uniprot.org/uniprotkb/Q5T2W1/entry) using domain 1 (aa 9-90) primers [5′-ATAAGAATGCGGCCGCGGAATGTA AACTGTCCAAGCAAGAA-3′ (sense) and 5′-CTCGACGATATCTTAATCCC CATCCAGAACTAGTAA-3′ (antisense)], domain 2 (aa 134-215) primers [5′-ATA AGAATGCGGCCGCGGCTCTGCTATCTCGTGAAGGAA-3′ (sense) and 5′-CTCGACGATATCTTATTCTTTGTCCACCAGCAGGAA-3′ (antisense)], domain 3 (aa 243-323) primers [5′-ATAAGAATGCGGCCGCGATACAATTCA AAAGAGAAACAGCC-3′ (sense) and 5′-CTCGACGATATCTTACTCTTTGTC TACCACCAA -3′ (antisense)], and domain 4 (aa 378-458) primers [5′-ATAA GAATGCGGCCGCGCTCTGCAGGCTGGCTAAAGGT-3′ (sense) and 5′-CTCG ACGATATCTTACTTCTTTCCACAGACTAGAAG-3′ (antisense)].

Each PDZ domain cDNA was digested with NotI and EcoRV and ligated into the p3xFlag plasmid.

All plasmids were confirmed by full-length sequencing using appropriate primers in the Einstein sequencing facility.

Preparation of Stably Transfected HeLa Cell Lines. HeLa cells (ATCC) stably transfected with pMEP4-hOATP1B1 or pMEP4-hOATP1B1$^{\Delta ETHC}$ were prepared using methods as we have described previously (Shi et al., 1995). In brief, cells were seeded in 10-cm polystyrene culture dishes (Corning #430167) in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech, Manassas, VA). All media were supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin–streptomycin (Mediatech), and cells were used for transfection at 70% to 80% confluence. Plasmid DNA (20 μg) mixed with transfection reagent (450 μL water, 50 μL 2.5 M $CaCl_2$, and 500 μL HEPES) was added to each culture dish containing 8 mL growth medium. Following an overnight incubation at 37°C in 5% CO_2, the medium was replaced, and cells were split 48 hours after transfection and cultured at 5% to 10% confluence in selective media containing 400 μg/ml Hygromycin B (Millipore). Two to three weeks later, colonies were picked and expanded in culture. Cells were harvested and lysed on ice for 30 minutes in PBS containing 1% Triton, 1 mM EDTA, and protease inhibitor [1 mM EDTA; 60 μM leupeptin; 105 μM AEBSF (4-benzenesulfonyl fluoride hydrochloride); 1 μM pepstatin A]. Lysates were centrifuged at 14,000 rpm for 30 minutes, and the supernatant was mixed with SDS sample buffer containing 0.1 M DTT and subjected to Western blot with antibody to hOATP1B1.

Antibodies and Reagents. A peptide was synthesized corresponding to amino acids 666–679 near the C-terminus of hOATP1B1 to which a cysteine was added at its C-terminus to enable linking to KLH. Inclusion of this cysteine did not change the specificity of this sequence for hOATP1B1 as determined by BLAST analysis. Rabbit antibody against this KLH-linked peptide was prepared in rabbits as we have described previously (Wang et al., 2003). The hOATP1B1 antiserum was immunopurified with peptide coupled to Sulfolink-agarose (Pierce) according to the manufacturer's instructions as we have described previously (Wang et al., 2005). Affinity purified rabbit IgG against hOATP1B3 was obtained from Sigma (HPA004943). Mouse monoclonal anti-hPDZK1 (sc-100337) was

obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Normal rabbit IgG was purchased from Sigma. ECL reagent for Western Blot analysis was obtained from PerkinElmer Life Sciences. Horseradish peroxidase–conjugated affinity-purified goat anti-rabbit IgG was obtained from Jackson ImmunoResearch (West Grove, PA). Mouse IgG kappa binding protein conjugated to horseradish peroxidase was obtained from Santa Cruz.

Uptake of ^{3}H-taurocholic Acid by HeLa Cell Lines. To determine whether loss of the PDZK1 consensus binding site influenced the transport function of hOATP1B1, uptake of ^{3}H-taurocholic acid (6.45 Ci/mmol; PerkinElmer, Shelton, CT) was determined in the HeLa cell lines expressing hOATP1B1 or hOATP1B1$^{\Delta ETHC}$. Previous studies showed that taurocholic acid is a transported substrate for hOATP1B1 (Mahagita et al., 2007). Cells (1 x 10^6) stably transfected with plasmids encoding hOATP1B1 or hOATP1B1$^{\Delta ETHC}$, were seeded in 60-mm cell culture dishes (Corning #430166) and cultured for 24 hours in medium containing 100 μM $ZnSO_4$ and then another 24 hours in 150 μM $ZnSO_4$ to induce transporter expression as we have described previously (Wang et al., 2003). At the time of study, cells expressing hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ were incubated in serum-free medium (SFM; 135 mM NaCl, 1.2 mM $MgCl_2$, 0.81 mM $MgSO_4$, 27.8 mM glucose, 2.5 mM $CaCl_2$, and 25 mM Hepes adjusted to pH 7.2) containing 1 μM ^{3}H-taurocholic acid at 37°C for 15 minutes after which they were washed 3 times with serum-free medium at 4°C. The third wash contained 5% bovine serum albumin and was allowed to stand for 5 minutes at 4°C; they were then harvested, and radioactivity was determined. Our previous studies revealed little uptake of ligands in cells incubated at 4°C for the duration of the study, and this was used to determine nonspecific uptake (Shi et al., 1995; Satlin et al., 1997). For technical reasons, expression of hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ could not be determined in the same plates that were used for uptake and were therefore quantified in triplicate in replicate plates by dot blot using the antibody to hOATP1B1 following a protocol provided by Abcam (Boston, MA; https://www.abcam.com/protocols/dot-blot-protocol). In brief, individual plates were washed with PBS, and cells were harvested. The cell suspensions were centrifugated at low speed (2000 rpm; 805 × g), and pellets were lysed by incubation for 30 minutes at 4°C with 70 μl of 1% Triton in PBS containing 1 mM EDTA and protease inhibitors. Lysate was then centrifuged at 14,000 rpm (20,800 × g) at 4°C for 30 minutes, and protein content of the supernatant was determined using the Pierce bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard. 1 μl (approximately 14 μg) of each supernatant was diluted 1:5 with lysis buffer, and 2 μl was spotted onto a nitrocellulose membrane (Bio Rad), air dried for 15 to 20 minutes, and processed as a Western blot using hOATP1B1 antibody. Expression of hOATP1B1 was quantified by densitometry and expressed as arbitrary units (AU) using ImageJ (National Institutes of Health public domain; http://rsb.info.nih.gov/ij/) as previously described (Choi et al., 2011). Specific cell-associated taurocholic acid was quantified as a function of transporter expression as pmoles of taurocholic acid/AU of hOATP1B1 or hOATP1B1$^{\Delta ETHC}$. Significance was determined using a two-tailed Student's *t* test.

Coupling of α-hOATP1B1, α-hOATP1B3, or normal rabbit IgG to agarose gel. IgG coupled to agarose gel was prepared as we described previously (Wang et al., 2005). Briefly, 0.04 mg of α-hOATP1B1 IgG, α-hOATP1B3 IgG, or normal rabbit IgG in 0.1 M borate buffer, pH 8.2, was rotated overnight at 4°C with 0.2 ml of protein A/G agarose (Santa Cruz, Dallas, Texas). Dimethyl pimelimidate 2HCl (20 mM final concentration) in 100 mM triethylamine in borate buffer was added, and the reaction was terminated after 60 minutes by the addition of 40 mM ethanolamine. The gel was washed with 0.2 M glycine/HCl at pH 2.3, followed by PBS, and stored at 4°C.

Immunoprecipitation from normal human liver membrane extracts. Normal human liver from a female subject was obtained from our Marion Bessin Liver Research Center institutional review board–approved biorepository (Taniguchi et al., 2020). Tissue was homogenized using a Bullet Blender Homogenizer (Next Advance, Inc., Troy, NY), as we described previously (Taniguchi et al., 2020). In brief, approximately 15 mg of liver in 1.0 mL PBS, pH 7.5 containing protease inhibitor was homogenized with 750 μg of 1.0-mm Zirconium Oxide Beads (Next Advance, Inc.) at a setting of 3 for 3 minutes and then at a setting of 12 for 5 minutes at 4°C. The homogenate was centrifuged at 350 × g for 6 minutes at 4°C, and the supernatant was suspended in 30 ml of 0.1 M Na_2CO_3 containing protease inhibitor for 15 minutes and centrifuged at 100,000 × g for 1 hour. The pellet was sonicated and resuspended in lysis buffer (1% Triton in PBS containing 1 mM EDTA and protease inhibitor) and incubated for 30 minutes on ice. After centrifugation at 14,000 rpm for 30 minutes at 4°C, total protein concentration was determined using the Pierce bicinchoninic acid kit (Pierce Biotechnology) with bovine serum albumin as the standard. Supernatant protein (800 μg) was incubated with 150 μl of protein A/G agarose coupled to α-hOATP1B1, α-hOATP1B3, or normal rabbit IgG and rotated at 4°C overnight. Gels were washed with lysis buffer, and immunoprecipitates were eluted from the gels with 0.2 M glycine, pH 2.3 into five 75 μl fractions. The acidic elutes were neutralized with 1M Tris-base and subjected to Western blot analysis for detection of hOATP1B1, hOATP1B3, and hPDZK1.

Pulldown Studies in Transiently Transfected 293T cells. 293T cells, obtained from the Cell and Tissue Core of the Marion Bessin Liver Research Center at the Albert Einstein College of Medicine, were used for transient co-transfection of N-Flag-hPDZK1 with sfGFP-hOATP1B1, sfGFP-hOATP1B1$^{\Delta ETHC}$, or sfGFP-hOATP1B3 as well as transient co-transfection of sfGFP-hOATP1B1 with N-p3x Flag-hPDZs (full-length and domains). In brief, 15 μg of each plasmid was mixed with 1 ml $CaCl_2$ transfection reagent and added to the cells for 48 hours. Cells were then washed with PBS, harvested, and pelleted at low speed (2000 rpm). Cells were lysed for 30 minutes on ice in lysis buffer containing protease inhibitor and then centrifuged at 20,000 x g for 30 minutes at 4°C. Supernatants were incubated overnight at 4°C with anti-flag M2 affinity agarose gel (Sigma), washed with lysis buffer, and eluted into five 50 μl fractions with 150 ng/μl of 3× Flag peptide (Glpbio Technology Inc. Montclair, CA). Eluates were subjected to Western blot analysis for detection of GFP and FLAG.

Immunofluorescence Analysis. Hela cells that had been stably transfected with hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ were plated onto eight-well cover glass bottom chambers (Nunc Laboratory-Tek cat. #155411) at various densities. Expression of hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ proteins was induced by the addition of 150 μM $ZnSO_4$ for 24 hours. As control, half of the wells were kept in the absence of $ZnSO_4$ to preclude hOATP protein expression. After the 24-hour induction or control period, the medium was removed, and cells were fixed for 10 minutes with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS and permeabilized with 0.01% saponin in PBS for an additional 10 minutes at room temperature. Cells were washed twice in PBS and blocked in 10% normal goat serum in PBS for 30 minutes and then incubated for 1 hour with affinity-purified primary antibody diluted 1:60 in blocking buffer. Cells were washed seven times and then incubated for 1 hour with goat anti-rabbit Cy5-labeled antibody (Jackson Immunoresearch, cat. #111-175-144) diluted 1:300 in blocking buffer and containing 10 μM Hoechst nuclear stain. Cells were then washed and imaged by spinning disk confocal microscopy utilizing a CARV II spinning-disk imager (Crisel Instruments, Rome, Italy) with DAPI, FITC, Rhodamine, and Cy5 wavelength filter settings, mounted to an Olympus IX71 microscope stand containing an iXon 897 EMCCD camera (Andor Technologies, Belfast Ireland), a PhotoFluor metal halide lamp (Chroma Technologies, Bellows Falls, VT), and an ×60 1.4 NA oil immersion objective lens all controlled by Metamorph software (Molecular Devices, San Jose CA) . Images were merged using FIJI/ImageJ (Schindelin et al., 2012), and graphics were added in Photoshop (Adobe Inc.). Control wells lacking incubation with primary antibody showed no fluorescence under these conditions.

Results

Determination of Association of hOATP1B1 and hOATP1B3 with hPDZK1

To test the hypothesis that hOATP1B1 is a ligand for hPDZK1, studies were performed in 293T cells in which FLAG-hPDZK1 was co-expressed with GFP-tagged full-length hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ lacking the C-terminal 4 amino acids (ETHC) that represent the PDZ consensus binding site. The experiment is diagramed in Fig. 1A. If full-length hOATP1B1 associates with hPDZK1 via its C-terminal PDZ consensus site, then Western blot of the FLAG immunoprecipitate of lysate from cells expressing GFP-hOATP1B1 will be positive for FLAG as well as GFP while lysate from cells expressing hOATP1B1$^{\Delta ETHC}$ will be positive only for FLAG. As seen in the representative study presented in Fig. 1B, this anticipated result was observed. That is, GFP-hOATP1B1 but not GFP-hOATP1B1$^{\Delta ETHC}$ was detected in immunoprecipitates of FLAG-hPDZK1. Although it is likely that binding of

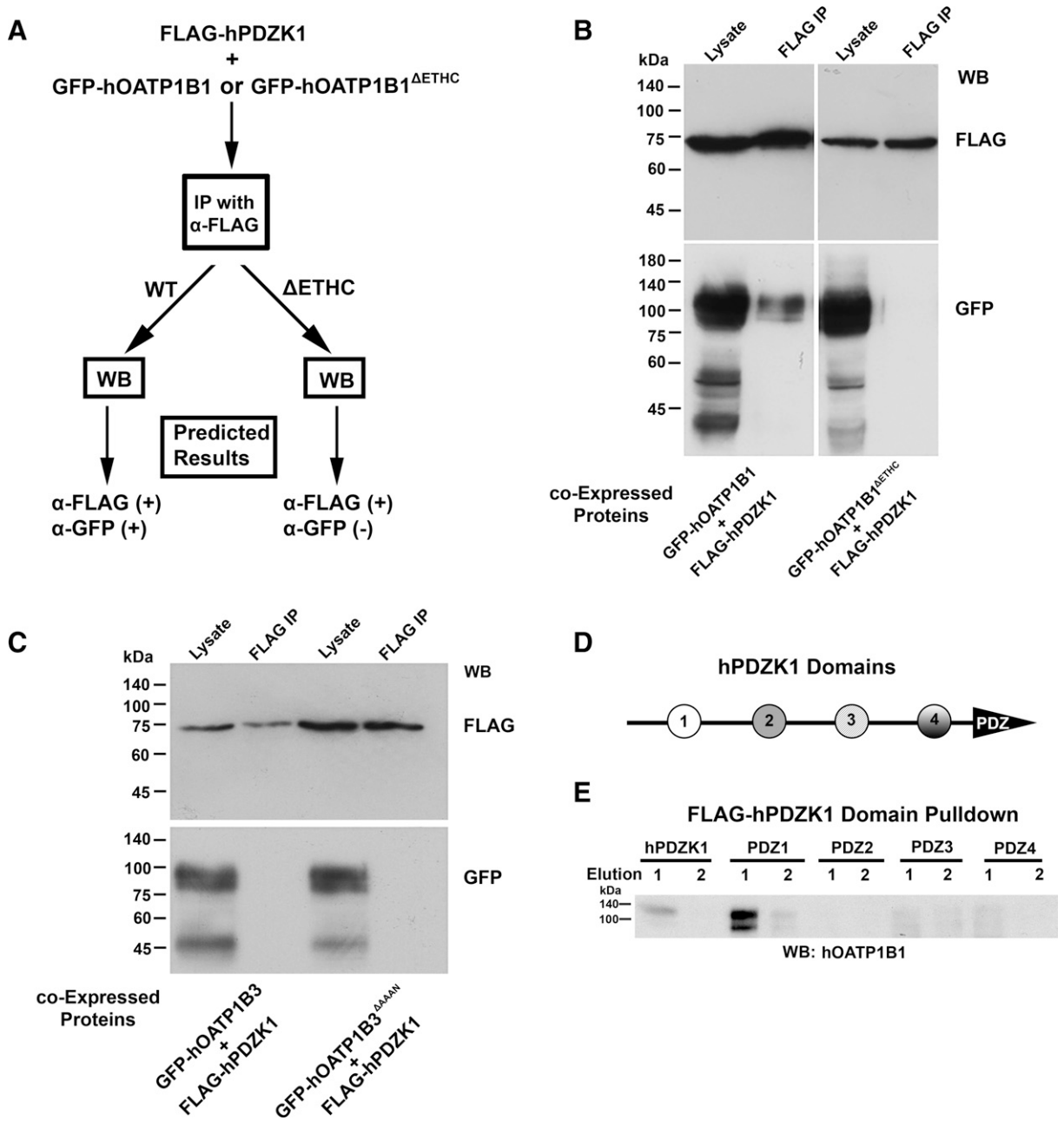

Fig. 1. hOATP1B1 is a ligand for hPDZK1. (A) Diagram of an experiment to determine whether hOATP1B1 binds to hPDZK1 and whether this requires the intact PDZ consensus binding site at its C-terminus. These studies were performed in 293T cells in which FLAG-hPDZK1 was co-expressed with GFP-tagged full-length hOATP1B1 or hOATP1B1 lacking the C-terminal 4 amino acids (ETHC) that represent the PDZ consensus binding site (hOATP1B1$^{\Delta ETHC}$). Immunoprecipitation with α-FLAG was performed as in Methods, and the presence of hOATP1B1 in the immunoprecipitate was determined by Western blot with α-GFP. If hOATP1B1 binds to hPDZK1 via its C-terminal 4 amino acid, hOATP1B1 should be present only in the immunoprecipitate from full-length hOATP1B1. (B) As seen in this representative study of three that were performed, in contrast to results for full-length hOATP1B1, hOATP1B1$^{\Delta ETHC}$ was not detected in the FLAG immunoprecipitate, consistent with the interpretation that hOATP1B1 is a ligand for hPDZK1 requiring the PDZ consensus site at its C-terminus. (C) A corresponding study was performed in cells transfected with GFP-hOATP1B3 or its truncated version, hOATP1B1$^{\Delta AAAN}$, lacking its C-terminal 4 amino acids (AAAN). In contrast to results with hOATP1B1, neither full-length nor truncated hOATP1B3 was found in the FLAG-hPDZK1 immunoprecipitate in this representative study of the three that were performed. (D) The domain organization of hPDZK1 is shown in schematic form. The full-length protein has four potential PDZ domains as well as a PDZ binding consensus site at its C-terminus (arrowhead). (E) FLAG pulldown studies were performed in 293T cells that were transiently co-transfected with GFP-hOATP1B1 and with FLAG constructs expressing full-length hPDZK1 or each of its four PDZ domains. The initial two of five 50μl eluate fractions from the FLAG immunoprecipitate are shown in this representative Western blot of two independent studies. PDZ1 was the only domain of hPDZK1 that bound hOATP1B1.

GFP-hOATP1B1 to hPDZK1 is direct, the possibility that hPDZK1 is pulled down with GFP-OATP1B1 through an intermediary protein that is co-immunoprecipitated by the OATP1B1 antibody must be considered. A corresponding study was performed in cells transfected with GFP-hOATP1B3 or its truncated version, hOATP1B3$^{\Delta AAAN}$, lacking its C-terminal 4 amino acids (AAAN). In contrast to results with hOATP1B1, neither full-length nor truncated hOATP1B3 was found in the FLAG-hPDZK1 immunoprecipitates (Fig. 1C). The domain organization of hPDZK1 is shown in schematic form in Fig. 1D. The full-length protein has four potential PDZ domains as well as a PDZ binding

consensus motif at its C-terminus (Anzai et al., 2004; Lalonde and Bretscher, 2009). To determine whether hOATP1B1 associates with specific domains of hPDZK1, additional pulldown studies were performed in 293T cells that were transiently co-transfected with GFP-hOATP1B1 and with FLAG constructs expressing full-length hPDZK1 or each of its four PDZ binding domains. as described in Methods. A representative Western blot is shown in Fig. 1E in which the first two eluate fractions from the anti-FLAG immunoprecipitation experiments were analyzed. As seen in the left-most lane, hOATP1B1 was found in the FLAG pulldown of lysate from cells expressing hOATP1B1 and full-length hPDZK1, similar to results in Fig. 1B. As seen in the remaining lanes, hOATP1B1 was only found in lysates that contained the PDZ1 domain of hPDZK1.

Interaction of hPDZK1 with hOATP1B1 in Human Liver

The preceding experiments showed that hOATP1B1 is associated with hPDZK1 but does not establish that it does so in intact liver. To examine this question, hOATP1B1 immunoprecipitates prepared from a Na_2CO_3 extract of normal human liver were examined for the presence of hPDZK1. Na_2CO_3 extraction enriches the content of integral membrane proteins such as OATPs as it removes more loosely associated proteins from the membranes as we have described previously (Bergwerk et al., 1996). Interestingly, we found that rPDZK1 remains associated with rOATP1A1 in Na_2CO_3 extracted rat liver membranes (Wang et al., 2005). Immunoprecipitation using nonimmune rabbit IgG was performed as a control. As described in Methods, immunoprecipitates were eluted from the immunoaffinity gels into five 75 μl fractions. These were subjected to Western blot analysis for detection of hOATP1B1 and hPDZK1. In the representative study in Fig. 2A, eluates 2 to 4 were analyzed by Western blot for detection of hOATP1B1 and hPDZK1. The nonimmune IgG immunoprecipitate did not contain hOATP1B1 and had little detectable hPDZK1. In contrast, both proteins were detected in the hOATP1B1 immunoprecipitate, consistent with their being associated with each other in the intact organ. Of note is the fact that the nonimmune IgG flow-through (FT) contained hOATP1B1 while the α-hOATP1B1 FT did not due to its depletion by the immunoaffinity gel. Both FTs contained hPDZK1. As seen in Fig. 2B, a corresponding study was performed with α-hOATP1B3. This showed abundant immunoprecipitation of hOATP1B3 but an absence of hPDZK1, indicating that hOATP1B3 is not associated with hPDZK1 in human liver, although the possibility must be considered that the antibody used for immunoprecipitation could block the binding site.

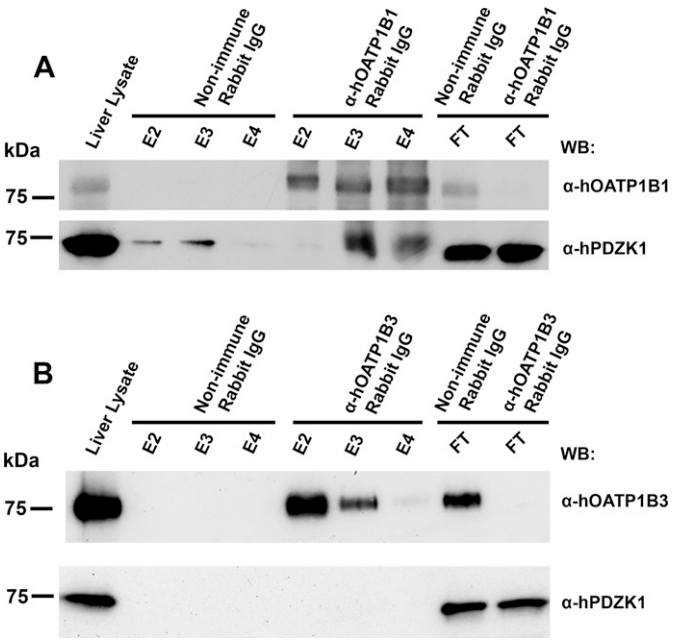

Fig. 2. hOATP1B1 but not hOATP1B3 interacts with hPDZK1 in human liver. To determine whether hOATP1B1 or hOATP1B3 is bound to PDZK1 in human liver, α-hOATP1B1 or α-hOATP1B3 immunoprecipitates were prepared from a Na_2CO_3 extract of normal human liver and probed by immunoblot for hPDZK1. Immunoprecipitation using nonimmune rabbit IgG was performed as a control. Immunoprecipitates were eluted from the immunoaffinity gels with 0.2 M glycine, pH 2.3 into five 75 μl fractions. The acidic elutes were neutralized with 1M Tris-base and subjected to Western blot analysis. In these representative studies of three that were performed, liver lysate, eluates 2 to 4, and the FT, which did not bind to the immunoaffinity gel, were analyzed by Western blot for detection of hOATP1B1 and hPDZK1 (A) and hOATP1B3 and hPDZK1 (B). hPDZK1 was found to be abundant in the hOATP1B1 immunoprecipitate but not in the nonimmune rabbit IgG or hOATP1B3 immunoprecipitates.

Interaction of hOATP1B1 with PDZK1 Regulates Its Subcellular Trafficking to the Cell Surface

Our previous studies showed that hPDZK1 is endogenously expressed in HeLa cells (Wang et al., 2019). In those studies, we showed that rOATP1A1 that has a PDZK1 consensus binding sequence at its C-terminus traffics to the plasma membrane of stably transfected HeLa cells. In contrast, rOATP1A4, which lacks a PDZK1 consensus site, remains largely intracellular in the absence of other OATPs (Wang et al., 2019). We now prepared HeLa cell lines that stably express full-length hOATP1B1 or the C-terminus truncated construct, hOATP1B1$^{\Delta ETHC}$, lacking the PDZK1 binding sequence. As noted in Methods, expression of both proteins was under regulation of a metallothionein promoter. As seen in the Western blot in Fig. 3A, in the absence of $ZnSO_4$ induction, there was little detectable hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ after 24 hours of culture. When cultured in the presence of $ZnSO_4$, there was abundant expression of both transporter forms. Distribution of both forms of hOATP1B1 was determined in these cells by immunofluorescence microscopy. As seen in the two representative fields in Fig. 3B, distribution of full-length hOATP1B1 was predominantly along the plasma membrane as indicated by the yellow arrows. In contrast, distribution of hOATP1B1$^{\Delta ETHC}$ was primarily intracellular with little seen along the plasma membrane. As expected, there was little signal in cells that were cultured in the absence of Zn (Fig. 3B, right-most panels). As an additional control, in the absence of primary antibody, cells that had been incubated in Zn showed no fluorescence (data not shown). Reduced surface expression of hOATP1B1$^{\Delta ETHC}$ correlated with reduced transport of ^{3}H-taurocholic acid as a function of transporter expression. In six independent studies, uptake by hOATP1B1 expressing cells was 0.31 ± 0.08 pmoles/AU, (mean ± standard error of the mean) versus 0.18 ± 0.04 pmoles/AU in hOATP1B1$^{\Delta ETHC}$ expressing cells ($P < 0.05$).

hOATP1B1 Interacts with hOATP1B3 in Human Liver

Whether hOATP1B1 and hOATP1B3 are bound to each other in liver was examined using a co-immunoprecipitation strategy. To confirm that the antibodies did not cross-react and were specific for their respective transporters, Western blots of lysates of 293T cells that had been transfected with expression plasmids encoding sfGFP-hOATP1B1 or sfGFP-hOATP1B3 were performed. As seen in Fig. 4A, the left-most panel shows that both GFP-linked transporters react with the α-GFP probe. The two panels to the right show that α-hOATP1B1 reacts with hOATP1B1 but not hOATP1B3 and that α-hOATP1B3 reacts with hOATP1B3 but not hOATP1B1. A Na_2CO_3 extract of normal human liver was subjected to immunoprecipitation using these antibodies or normal rabbit IgG as a control. Immunoprecipitates were eluted from the agarose gel into five fractions and subjected to Western blot as

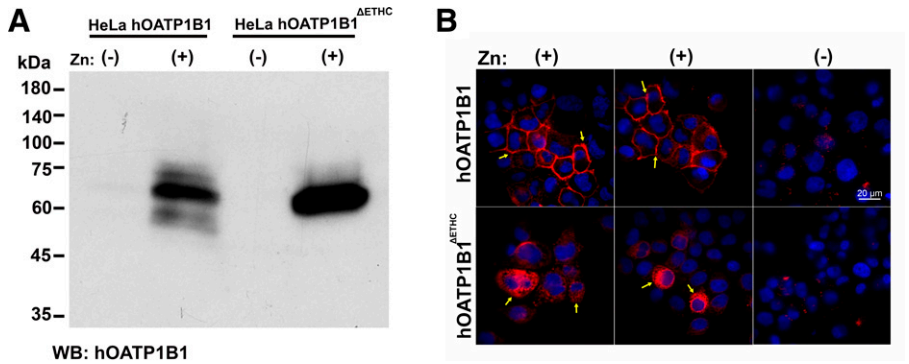

Fig. 3. Immunofluorescence analysis of the subcellular distribution of hOATP1B1 and hOATP1B1$^{\Delta ETHC}$ expressed in stably transfected HeLa cells that endogenously express hPDZK1. (A) 1% Triton X-100 lysates of stably transfected HeLa cell lines expressing hOATP1B1 or hOATP1B1$^{\Delta ETHC}$ under regulation by a metallothionein promoter were prepared as in Methods and were incubated for 24 hours with or without 150 μM $ZnSO_4$ added to the culture medium. hOATP1B1 expression was examined by immunoblot. In the absence of Zn induction, there was little detectable hOATP1B1 or hOATP1B1$^{\Delta ETHC}$. When cultured in the presence of Zn, there was abundant expression of both transporter forms. (B) Distribution of both forms of hOATP1B1 was determined in Zn-induced cells by immunofluorescence microscopy. Two representative fields of multiple studies for each expressed protein are shown. As indicated by the yellow arrows in the top panels, full-length hOATP1B1 was distributed predominantly along the plasma membrane. In contrast, as seen in the lower panels, distribution of hOATP1B1$^{\Delta ETHC}$ was primarily intracellular, with little seen along the plasma membrane. As seen in the right-most panels, there was little signal in cells that were cultured in the absence of Zn, in agreement with lack of expression seen by Western blot.

described in Methods. A representative study is shown in Fig. 4B in which eluates 2 to 4 were analyzed for each condition. There was no reactivity to either antibody in fractions from normal rabbit IgG. In contrast, hOATP1B1 immunoprecipitates contained hOATP1B3, and hOATPB3 immunoprecipitates contained hOATP1B1.

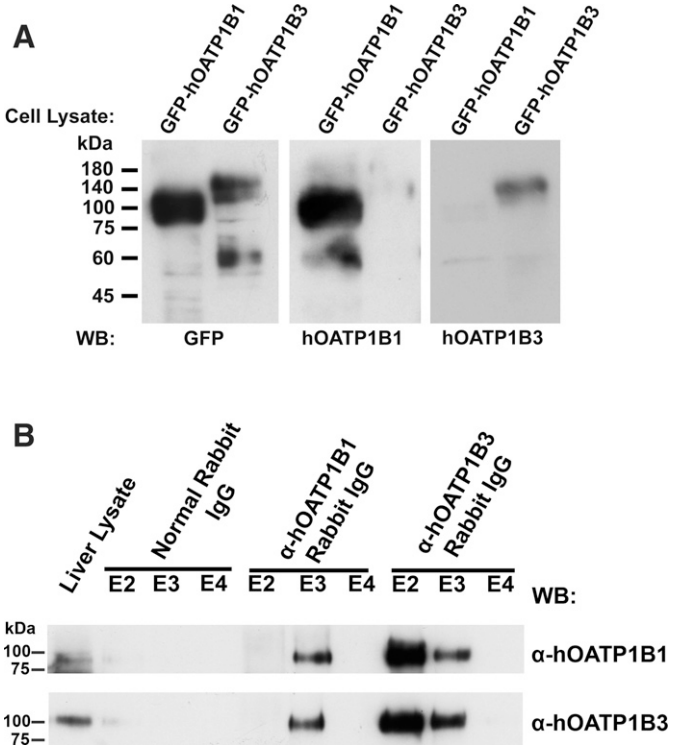

Fig. 4. hOATP1B1 and hOATP1B3 interact in human liver. (A) Specificity of antibodies to hOATP1B1 and hOATP1B3 was determined by Western blot of lysates of 293T cells that transiently expressed plasmids encoding sfGFP constructs of each of the transporters. The left-most panel shows that both lysates reacted with antibody to GFP. The middle panel shows that the antibody to hOATP1B1 did not react with hOATP1B3, and the right-most panel shows that the antibody to hOATP1B3 did not react with hOATP1B1. (B) In a representative study of four that were performed, both hOATP1B1 and hOATP1B3 were present in immunoprecipitates from either hOATP1B1 (top panel) or hOATP1B3 (bottom panel) from a Na_2CO_3 extract prepared from human liver, indicating that these proteins interact. Neither protein was detected in the normal rabbit IgG immunoprecipitate.

Discussion

Since the initial discovery of OATP, now known as rOATP1A1, in rat liver in 1994 (Jacquemin et al., 1994), it has been recognized that it is a member of a highly related superfamily of organic anion-transporting polypeptides numbering over 300 members in over 40 species (Hagenbuch and Stieger, 2013; Schulte and Ho, 2019). Many members of this OATP superfamily are expressed in the liver and mediate uptake of a diverse number of amphipathic compounds (Hagenbuch and Stieger, 2013; Wolkoff, 2014; Schulte and Ho, 2019). In previous studies, we found that rOATP1A1 contains a PDZ binding consensus sequence that is defined by the four amino acids (KTKL) at its C-terminus. Further studies revealed that rOATP1A1 associates with rPDZK1, a well-characterized scaffolding protein with four independent potential binding domains. rOATP1A1 was found to associate with domains 1 and 3 (Wang et al., 2005), and this association is required for trafficking of the transporter to the hepatocyte surface (Wang et al., 2005, 2014; Choi et al., 2011). In the absence of rPDZK1, rOATP1A1 resides largely in an intracellular vesicular pool with little on the cell surface, and transport function is reduced accordingly (Wang et al., 2005). Interestingly, when rPDZK1 is present on these intracellular vesicles, they recruit specific microtubule-based motor proteins that can direct their movement toward the cell surface (Wang et al., 2014). Although all members of the OATP family that are expressed in liver are found on the plasma membrane, many, such as rOATP1A4, do not have a PDZ consensus binding site (Anwer and Wolkoff, 2020). In recent studies, we found that rOATP1A4 can associate with rOATP1A1 and traffic as a complex with rPDZK1 through the hepatocyte to the plasma membrane (Wang et al., 2019).

In human liver, hOATP1B1 and hOATP1B3 play a major role in xenobiotic transport (Gong and Kim, 2013; Zhang et al., 2017; Schulte and Ho, 2019). Although they are highly expressed on the basolateral plasma membrane of hepatocytes, it has been thought that neither of these transporters contains a PDZ consensus binding motif (Kato et al., 2004; Anwer and Wolkoff, 2020). However, computer algorithm–based analysis of hOATP1B1 suggests that its C-terminal amino acid sequence (ETHC) is, in fact, a putative ligand for hPDZK1 (Hui et al., 2013; Wang et al., 2019). Analysis of hOATP1B3 using this algorithm finds no evidence for a PDZ consensus sequence, in agreement with past biochemical studies (Kato et al., 2004). Although previously unsuspected, interaction of hOATP1B1 with hPDZK1 can have important physiologic consequences, and confirmation of this interaction was

obtained in the present study. As we have described previously for rOATP1A1 (Wang et al., 2005, 2014, 2019), interaction with hPDZK1 is required for optimal expression of hOATP1B1 on the cell surface (Fig. 3B). Although hOATP1B3 does not have a PDZ consensus binding motif, it is still found localized to the hepatocyte basolateral plasma membrane. Interesting studies from the Hagenbuch laboratory have shown that hOATP1B3 can oligomerize with hOATP1B1 as well as with several other transporters (Zhang et al., 2017, 2020). This has been confirmed in the present study in which we found that these two transporters co-immunoprecipitate from human liver. These studies were performed with liver from a single normal subject, and the physiologic importance remains to be determined. However, we suggest that hOATP1B3 may traffic through the hepatocyte as a complex with hOATP1B1 analogous to what was described for the rat transporters rOATP1A1 and rOATP1A4 (Wang et al., 2019).

Cell surface expression of these transporters is necessary for them to mediate ligand uptake, and it is clear that assays that determine total protein or mRNA expression in liver need to be interpreted with this in mind (Wang et al., 2005; Murray and Zhou, 2017). A number of polymorphisms of the hOATPs that reduce their expression or transport function have been described (Nakanishi and Tamai, 2012; Gong and Kim, 2013). There has been little work regarding polymorphisms of proteins such as PDZK1 that may modulate the subcellular trafficking and processing of the OATPs. As previously noted, PDZK1 is a scaffold protein that plays a role in trafficking of plasma membrane–associated proteins in various tissues throughout the body. Among these transporters is the renal urate-anion exchanger URAT1. URAT1 has a PDZK1 consensus binding site defined by its C-terminal amino acids (STQF) and binds to PDZ domains 1, 2, and 4 of PDZK1 (Anzai et al., 2004). Significantly, specific polymorphisms of hPDZK1 correlate with the occurrence of gout in Chinese (Li et al., 2015) and Japanese (Higashino et al., 2016) populations, presumably due to altered URAT1 trafficking. Similarly, the HDL scavenger receptor class B type 1 is a ligand for hPDZK1, requiring interaction for trafficking and hepatocellular expression. Several polymorphisms of hPDZK1 have been associated with occurrence of metabolic syndrome in these patients (Junyent et al., 2009). Conversely, overexpression of hPDZK1 could augment transporter trafficking to the plasma membrane, which may be an important factor in development of drug resistance in tumors (Inoue et al., 2004).

The fact that murine and human OATPs use PDZK1 for plasma membrane targeting suggests that this mechanism may be used by other species, including dog and pig, which have been important models for pharmacologic study. Canine OATP1B4, the major organic anion transporter in canine liver (Martinez et al., 2021), has a potential PDZK1 binding consensus sequence (ETHM) at its C-terminus (Gui and Hagenbuch, 2010). However, it is not clear that the C-terminal amino acids of the corresponding porcine OATP1B3 (ETHI) will interact with porcine PDZK1, and further studies will be required (Kojima and Degawa, 2022). Interestingly, porcine OATP1A2 has the same terminal amino acid sequence (KTKL) as rat and mouse OATP1A1 as well as human OATP1A2 all of which interact with PDZK1. These studies suggest that PDZK1 regulation of OATP transport activity is conserved across species and may be multifactorial, involving subcellular targeting and homo- and hetero-oligomerization that may influence substrate specificities. Microtubule-based trafficking of transporter-containing endocytic vesicles may be an important aspect of these regulatory processes (Wang et al., 2014). Future study of this underexplored field may yield important insights regarding interindividual heterogeneity in drug bioavailability and toxicity.

Data Availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Authorship Contributions

Participated in research design: Wang, Murray, Wolkoff.
Conducted experiments: Wang, Murray.
Contributed new reagents or analytic tools: Wang, Murray.
Performed data analysis: Wang, Murray, Wolkoff.
Wrote or contributed to the writing of the manuscript: Wang, Murray, Wolkoff.

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