Transmembrane Domain 1 of Human Organic Anion Transporting Polypeptide 2B1 Is Essential for Transporter Function and Stability

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ABSTRACT

Organic anion transporting polypeptides (OATPs, gene symbol SLCO) are important membrane transporter proteins that mediate the uptake of wide ranges of endogenous and exogenous compounds. OATP2B1 has been found in multiple organs and tissues, including the liver, small intestine, kidney, brain, placenta, heart, skin, as well as skeletal muscle, and is proposed to be involved in the uptake of orally administered drugs. Quite a few reports have demonstrated that transmembrane domains (TMs) are crucial for proper functions of OATP family members. Comparative modeling proposed that TM1, along with TM2, 4, and 5 of the N-terminal half of OATP2B1, may be localized within the substrate interaction pocket and are important for uptake function of the transporter. Alanine scanning of the putative transmembrane domain 1 of OATP2B1 revealed that substitution of L58 with alanine dramatically altered the $K_m$ value, and mutation of V52, H55, Q59, and L69 resulted in significantly reduced substrate turnover number, whereas A61V, Q62A, and S66A exhibited significant change in both $K_m$ and $V_{max}$ values. In addition, phenylalanine at position 51 seems to play an important role in maintaining proper folding of OATP2B1 because alanine replacement of F51 caused accelerated degradation of the transporter protein. Although proteasome and lysosome inhibitors could partially recover protein level, the mutant transporter remained nonfunctional. Taken together, the identification of nine essential amino acid residues within TM1 of OATP2B1 suggested that the transmembrane domain is important for maintaining proper function of the transporter.

Introduction

Organic anion transporting polypeptides (OATPs, gene symbol SLCO) belong to the solute carrier family and mediate sodium-independent transport of various endogenous and exogenous compounds (Hagenbuch and Gui, 2008). Bile salts, hormones and their conjugates, toxins, and a wide range of drugs have been found to be transported by OATPs. Twelve human OATP family members have been found so far: OATP1A2, 1B1, 1B3, 1B7, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1, and 6A1 (Hagenbuch and Meier, 2003; Nakaniishi and Tamai, 2012). However, SLCO1B7 was proposed as a pseudogene because OATP1B7 is considered as nonfunctional (Stieger and Hagenbuch, 2014). Some OATP family members are predominantly expressed in certain organs or tissues. For example, OATP1B1 and OATP1B3 are found only in the liver, whereas others were reported as being expressed ubiquitously (König et al., 2006). Due to their broad substrate specificity, wide tissue distribution, and the involvement of drug-drug interactions, OATPs have been extensively recognized as key determinants for drug absorption, distribution, and excretion (Shitara et al., 2005; Poirier et al., 2007).

OATP2B1 has been found in multiple organs and tissues, including the liver, small intestine, kidney, brain, placenta, heart, skin, as well as skeletal muscle (Tamai et al., 2000; Kullak-Ublick et al., 2001; Hagenbuch and Meier, 2004; Niessen et al., 2009; Knauer et al., 2010, 2013). In the liver, OATP2B1 is involved in hepatic uptake of a wide spectrum of xenobiotics, and many organic anions, mono- and dicarboxylic acids (Kobayashi et al., 2003; Sai et al., 2006), steroid hormones and their derivatives (Grube et al., 2006), as well as drugs such as rifamycin SV (Vavricka et al., 2002), pravastatin (Kobayashi et al., 2003), cyclosporine, and gemfibrozil (Tamai et al., 1997) are inhibitors of the transporter. OATP2B1 exhibits high expression in the apical membrane of human intestinal epithelial cells and may function as a pH-dependent organic anion transporter (Kis et al., 2010). Since the physiologic microclimate pH in the intestinal lumen is weakly acidic, OATP2B1 that shows higher activity at acidic pH is believed to be involved in the uptake of drugs administered orally (Kobayashi et al., 2003; Tamai, 2012). Although extensive studies have been carried out to identify substrates of OATPs, the underlying mechanisms of substrate binding

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ABBREVIATIONS: ES, estrone-3-sulfate; MG132, carbenoxoxy-Leu-Leu-leucinal; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; OATP, organic anion transporting polypeptide; TM, transmembrane domain.

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Transmembrane Domain 1 Is Important for OATP2B1 Function

Transmembrane domains (TMs) have been demonstrated to be important for proper functions of various transporters. Previous studies have identified quite a few essential amino acids located within transmembrane domains of OATP members. For example, amino acid residues within TM2 (Li et al., 2012), TM6 (Huang et al., 2013), TM10 (Gui and Hagenbuch, 2009; Ohnishi et al., 2014), and TM11 (Weaver and Hagenbuch, 2010; Hong et al., 2015) were shown to be crucial for proper function of OATP1B1, and TM8 (Miyagawa et al., 2009) is believed to be important for substrate recognition of the transporter. As for OATP1B3, K41 in TM1 and R580 in TM11 are believed to be pivotal to the transporter function (Glaeser et al., 2010). In addition, G45 within TM1 along with Y537, SS45, and T550 within TM10 (Gui and Hagenbuch, 2008; DeGorter et al., 2012) were demonstrated to be critical for the transport of OATP1B3-specific substrate CCK8. TM6 of OATP1A2 seems to be an important region for substrate binding, protein stability, and trafficking (Chan et al., 2015). However, little information is available for the role of TMs within OATP2B1.

Comparative modeling proposed that transmembrane helix 1 along with TM2, 3, and 5 of the N-terminal half of OATP2B1 may be involved in formation of the substrate interaction pore (Meier-Abt et al., 2005). In the present study, alanine scanning of the putative TM1 of OATP2B1 identified nine amino acid residues that are essential for the transporter function. Kinetic analysis of the functionally impaired mutants showed that Km and/or Vmax values of most of the mutants were affected, and that F51A exhibited dramatically reduced protein expression. The identification of nine critical amino acid residues within TM1 of OATP2B1 suggested that the transmembrane domain is important for maintaining proper function of the transporter protein.

Materials and Methods

Materials. [3H]Estrone-3-sulfate (ES) and [3H]taurocholic acid were obtained from PerkinElmer Life Sciences (Waltham, MA). Sulfo succinimidyl 2-(bisthioato)-ethyl-1,3-dithiopropionate (NHSS-biotin) and streptavidin-agarose beads were purchased from Thermo Scientific (Rockford, IL). All other reagents were from Sigma-Aldrich (St. Louis, MO) except when otherwise stated.

Site-Directed Mutagenesis. Mutants were generated using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). The pReceiver M07 vector that contains the SLCO2B1 cDNA and 3-hemagglutinin tags at the C terminus was obtained from GeneCopoeia (Rockville, MD) and used as a template for the mutagenesis. All mutant sequences were confirmed by full-length sequencing (Thermo Scientific).

Cell Culture and Transfection of Plasmid Constructs into Cells. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Thermo Scientific) supplemented with 10% fetal bovine serum at 37°C and 5% CO2. Confluent cells in a 48-well or six-well plate were transfected with DNA plasmid using Lipofectamine 2000 reagent (Thermo Scientific) following the manufacturer’s instructions and used for transport assay and cell surface biotinylation 48 hours after transfection.

Cell Surface Biotinylation and Western Blotting. Cell surface expression level of OATP2B1 and mutants was examined using the membrane-impermeable biotinylation reagent NHS-SS-biotin as described before (Li et al., 2012; Huang et al., 2013). In brief, HEK293 cells expressing OATP2B1 or mutants were labeled on ice with NHS-SS-biotin in two successive 20-minute incubations and lysed with radioimmunoprecipitation assay buffer [50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40, and protease inhibitors phenylmethylsulfonyl fluoride (200 μg/ml) and leupeptin (3 μg/ml), pH 7.4]. The labeled proteins in supernatant were then pulled down by streptavidinagarose beads, released in 4× Laemmli buffer and loaded onto a 7.5% SDS–polyacrylamide electrophoresis gel, transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and detected with anti-hemagglutinin antibody (Cell Signaling Technology, Danvers, MA).

Uptake Assay. HEK293 cells in a 48-well plate were used for transport measurement as described before (Li et al., 2012; Huang et al., 2013) with minor modification. In brief, cells were incubated with uptake solution that contained [3H]ES (pH 7.4) or [3H]taurocholic acid (pH 5.0) at 37°C for 2 minutes (1 minute for kinetic analysis), and the reaction was stopped by ice-cold phosphate-buffered saline solution. Cells were then washed twice with cold phosphate-buffered solution, solubilized in 0.2 N NaOH followed by neutralization with 0.2 N HCl, and radioactivity of the cell lysate was measured using a Triathler-Hidex (Hidex, Turku, Finland) liquid scintillation counter. The uptake count was standardized by the amount of protein in each well.

Statistical Analysis. Data statistical analysis was carried out using one-way analysis of variance with Bonferroni’s post hoc test. Differences between means are regarded as significant if P < 0.05.

Results

Characterization of OATP2B1 Function in HEK293 Cells. To see whether OATP2B1 could be properly expressed and function in HEK293 cells, we first analyzed cell surface expression and kinetic parameters of OATP2B1. As shown in Fig. 1, OATP2B1 was expressed and correctly targeted to the plasma membrane and exhibited a Km value of 6.76 ± 0.71 μM, which is consistent with the previously reported Km of 8.09 ± 1.67 μM (Nozawa et al., 2004) for OATP2B1 expressed in HEK293 cells. These results indicated that OATP2B1 was properly expressed and functioned within HEK293 cells.

Effect of TM1 Mutants on OATP2B1 Uptake Function and Protein Expression. To analyze critical amino acid residues within TM1, we performed alanine scanning. Each of the amino acid residues located within putative transmembrane domain 1 of OATP2B1 (Fig. 2) was mutated to alanine, and uptake function of each mutant was measured. Interestingly, most of the alanine mutants showed statistically significant change of ES uptake, and more than half of the mutants (11 out of 21) exhibited greater than 50% reduction of ES uptake function (P < 0.05) with the exception of F51A, all mutants exhibited cell surface expression level of OATP2B1 and mutants was examined using the membrane-impermeable biotinylation reagent NHS-SS-biotin as described before (Li et al., 2012; Huang et al., 2013). In brief, HEK293 cells expressing OATP2B1 or mutants were labeled on ice with NHS-SS-biotin in two successive 20-minute incubations and lysed with radioimmunoprecipitation assay buffer [50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40, and protease inhibitors phenylmethylsulfonyl fluoride (200 μg/ml) and leupeptin (3 μg/ml), pH 7.4]. The labeled proteins in supernatant were then pulled down by streptavidin-agarose beads, released in 4× Laemmli buffer and loaded onto a 7.5% SDS–polyacrylamide electrophoresis gel, transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and detected with anti-hemagglutinin antibody (Cell Signaling Technology, Danvers, MA).

Kinetic Analysis of TM1 Mutants with Significantly Reduced Estrone-3-Sulfate Uptake Function. To evaluate whether the effect on uptake function of these mutants was due to reduced protein level on the plasma membrane, ES uptake of different mutants was normalized with their cell surface expression level (Fig. 4C). It was shown that after
protein level adjustment, ES uptake of K49A, F51A, and I65A was partially recovered to more than 50% of that of wild-type OATP2B1, suggesting that these residues may have affected protein expression of the transporter, which in turn led to reduced uptake function. On the other hand, the other eight mutants, i.e., V52A, H55A, L58A, Q59A, A61V, Q62A, S66A, and L69A, still exhibited more than 50% decrease of transport activity, indicating that replacement of these residues may change the interaction of the transporter with substrate. Therefore, kinetic analysis of ES uptake was performed, and as shown in Table 1, A61V, Q62A, and S66A affected binding affinity as well as substrate turnover number of the transporter; whereas L58A only showed increased Km value, V52A, H55A, Q59A, and L69A only showed altered Vmax.

To see whether only ES uptake was affected by mutation at these positions, we analyzed transport activity of taurocholate by OATP2B1 and the functionally impaired mutants as well. As shown in Fig. 5, mutants that showed more than 50% decrease in ES uptake also exhibited significant reduction (>50%) of transport activity for taurocholate, suggesting these residues are involved in uptake of both substrates.

**Alanine Replacement of F51A Affects Protein Stability of OATP2B1.** To see whether the significantly reduced expression of F51A was due to an increased degradation of

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Fig. 1. Characterization of OATP2B1 in HEK293 cells. (A) Cell surface (upper panel) and total protein (lower panel) expression of OATP2B1 in HEK293 cells. Cells were biotinylated and lysed with radioimmunoprecipitation assay buffer, and the biotin-labeled cell surface proteins were then precipitated with streptavidin-agarose beads, separated by SDS-PAGE, followed by western blotting with anti-hemagglutinin antibody. Or cells were lysed with radioimmunoprecipitation assay buffer and subjected to analysis as described earlier. The same blot was probed with integrin or actin as protein loading control. (B) Kinetic analysis of OATP2B1. Uptake (pH 7.4) of ES was measured at concentrations ranging from 0.05 to 100 μM at 37°C at a 1-minute interval. The results represent data from three experiments, with duplicate measurements for each sample. The results shown are means ± S.D. (n = 3). Km (shown as mean ± S.D.) was determined with nonlinear regression of the Michaelis-Menten equation incorporated in GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Fig. 2. Sequence comparison of OATP family members. Multiple sequence alignment of different OATPs was performed with Clustal W (www.clustal.org). The putative transmembrane domain 1 of OATP family members was predicted according to the Kyte-Doolittle hydrophobicity scale. Only partial sequences are shown. The corresponding sequences of TM1 are in bold.
the transporter, the mutant was treated with proteasome inhibitor MG132 (carbobenzoxy-Leu-Leu-leucinal) or lysosome inhibitor bafilomycin A1. As shown in Fig. 6A, MG132 treatment resulted in partial recovery of the total protein (left panel). In addition, it was observed that only the immature form (∼72 kD) of the transporter was recovered after MG132 treatment; the mature form (∼100 kD) of the protein remained almost undetectable. Consistent with this result, cell surface expression of OATP2B1 and its alanine-substituted mutants was measured at 37°C at a 2-minute interval. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from cells expressing wild-type OATP2B1 (OATP2B1-WT) or mutants. The results represent data from three experiments, with duplicate measurements for each sample. The results shown are means ± S.D. (n = 3). Asterisks indicate significant difference compared with wild-type OATP2B1 (P < 0.05).
substitution of Q62 with asparagine exhibited decreased function and protein expression of the transporter, whereas replacement of H55 with lysine significantly reduced uptake transport activity (Fig. 7). On the other hand, conservative hydrophobic residues (V52, L58, and L69) with an amino acid conservative amino acids. As shown in Fig. 7, replacement of for OATP2B1 function, we substituted the residues with side chain structures of these critical amino acids are essential for OATP2B1 Function.

To investigate whether the transport function of F51A remained negligible even after MG132 treatment (right panel). Accordingly, MG132 treatment showed a marginal effect on uptake function of F51A (Fig. 6B). Treatment with the vacuolar proton ATPase inhibitor bafilomycin A1 resulted in a significantly elevated level of F51A (Fig. 6C). Further analysis revealed that the lysosome inhibitor bafilomycin A1 resulted in a significantly elevated level of F51A expression (Table 1). After normalization with cell surface expression, Km and Vmax of F51Y were comparable to those of wild-type OATP2B1, indicating that phenylalanine at this position may not directly interact with the substrate (Table 1).

**Discussion**

Although quite a few reports have shown that OATP2B1 is involved in the transport of a wide range of compounds, information related to the structure-function relationship of OATP2B1 is still limited. OATP2B1 has been demonstrated to contain multiple substrate binding sites in Xenopus oocytes (Shirasaka et al., 2012) and Caco-2 cells (Kis et al., 2010). However, in our current study, the transporter only showed monophasic transport of estrone-3-sulfate. The difference may be attributable to the different systems used. In an earlier study of OATP2B1 expressed in HEK293 cells, it was shown that the transporter exhibited single saturation kinetics as well (Nozawa et al., 2004).

Transmembrane domains have been demonstrated to play important roles in proper function of OATP1B1, 1B3, and 1A2. In the present study, we performed alamine scanning of putative TM1 of OATP2B1 and found that alanine substitution of 11 residues within the transmembrane domain resulted in more than 50% reduction of ES uptake by the transporter. After normalization with cell surface protein level, there were still eight mutants exhibiting greatly reduced transport function (>50%, Fig. 4C). When locations of these essential residues were analyzed, it was found that from V52 on, alanine replacement of every three and/or four residues, i.e., V52, H55, L58, Q59, A61, Q62, S66, L69, resulted in a mutant that showed significantly decreased transport function. Since amino acid residues spaced three or four residues apart in protein primary sequence are spatially close to each other in an α-helix (Tymoczko et al., 2015), these TM1 residues may be lined up at the same side of the α-helix, interacting with OATP2B1 substrates. A similar phenomenon was observed in our previous study of OATP1B1. It was found that several amino acid residues along TM11 are crucial for uptake function of the transporter, and they are located three and/or four residues apart from each other (Hong et al., 2015).

**Table 1**

Kinetic parameters of estrone-3-sulfate transport by wild-type OATP2B1 and TM1 mutants

<table>
<thead>
<tr>
<th>Residue</th>
<th>Km (μM)</th>
<th>Vmax (pmol/mg protein/min)</th>
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<tr>
<td>OATP2B1</td>
<td>6.40 ± 0.30</td>
<td>351 ± 1</td>
</tr>
<tr>
<td>F51Y</td>
<td>7.30 ± 1.70</td>
<td>365 ± 6</td>
</tr>
<tr>
<td>V52A</td>
<td>5.28 ± 1.54</td>
<td>71.4 ± 3.4*</td>
</tr>
<tr>
<td>H55A</td>
<td>6.75 ± 1.17</td>
<td>88.5 ± 2.0*</td>
</tr>
<tr>
<td>L58A</td>
<td>18.4 ± 2.6*</td>
<td>314 ± 29</td>
</tr>
<tr>
<td>Q59A</td>
<td>6.27 ± 0.65</td>
<td>27.9 ± 3.1*</td>
</tr>
<tr>
<td>A61V</td>
<td>24.9 ± 4.2*</td>
<td>213 ± 20*</td>
</tr>
<tr>
<td>Q62A</td>
<td>7.36 ± 1.01*</td>
<td>134 ± 1*</td>
</tr>
<tr>
<td>S66A</td>
<td>10.1 ± 0.7*</td>
<td>167 ± 4*</td>
</tr>
<tr>
<td>L69A</td>
<td>7.20 ± 1.30</td>
<td>75.3 ± 4.8*</td>
</tr>
</tbody>
</table>

*Values significantly different (P < 0.05) from that of wild-type OATP2B1.
Indeed, homology modeling (Biasini et al., 2014) of OATP2B1 (Supplemental Material) with *Escherichia coli* glycerol-3-phosphate transporter (Protein Data Bank: 1pw4, Supplemental Material) as template revealed that V52, H55, L58, Q59, A61, Q62, S66, and L69 are all localized along one side the α-helix, facing the inner channel formed among different transmembrane domains (Fig. 8). These results are consistent with previous reports of putative computer models of OATP family members. It was proposed that for OATP1B3, the N-terminal half of TMs 1, 2, 4, and 5 and the C-terminal half of TMs 7, 8, 10, and 11 likely face the central pore, and that the presence of a central pore may be a conserved feature and of functional significance for OATP members (Meier-Abt et al., 2005). A more recent structural model of OATP2B1 also revealed that amino acid residues of TMs 1, 2, 4, 5, 7, 8, 10, and 11 may be involved in the formation of the putative substrate translocation pathway of the transporter (Bian et al., 2016). It should be noted that the sequence identity of glycerol-3-phosphate transporter compared with OATP2B1 is relatively low (12.87%). However, homology modeling using the transporter covers the most extended area, i.e., from TM1 to TM12 of OATP2B1, among different templates with comparable identity. A similar structure was obtained using the human glucose transporter family member 1 (Protein Data Bank: 4pyp), which has the highest sequence identity (15.82%) compared with OATP2B1 but exhibits a lower coverage along the sequence (data not shown) as template.

Among the identified residues, V52 is highly conserved, with 10 OATPs having a nonpolar, hydrophobic amino acid at this position (Fig. 2). At position L58, other OATPs except the two OATP1B family members have the same residue or a residue with similar structure. Similarly, the residues at position 69 are nonpolar and hydrophobic in OATP2B1 and other OATPs, with the exception of OATP1B1, 1B3, and 1A2. The conservative replacement of these residues partially recovered uptake function of the transporter, suggesting the hydrophobic property of these residues may be important for transport function of OATP members. A61 seems to be only conserved among OATP2B1 and most of the OATP1 subfamily members, whereas a polar residue is found in other OATPs. At position Q62, the residue is conserved in other OATPs except those of the OATP1 family, which all contain a positively charged lysine residue at this position. On the other hand, H55, Q59, and S66 seem to be unique for OATP2B1 and may be involved in OATP2B1-specific functions.

The protein expression of F51A was dramatically reduced, suggesting phenylalanine at this position may play an important role in protein stability. Proteasome inhibitor MG132 partially recovered the immature form of the transporter (~72 kD), whereas lysosomal inhibitor bafilomycin A1 increased both the immature and mature forms (~100 kD) of F51A, and the cell surface expression of the transporter was partially recovered. However, the recovered F51A is not functional. Both the proteasome and lysosome are important sites for
the degradation of misfolded proteins (Ciechanover, 2005).
These data suggested that alanine replacement of F51 may
result in a protein that is recognized by the quality control
machinery as incorrectly folded and hence targeted for
degradation by the proteasome and lysosome. However,
mutation of F51 does not seem to affect maturation of the
protein because blocking the lysosomal pathway could par-
tially recover the mature form of the transporter. Conserva-
tive replacement with tyrosine resulted in partial recovery of
transport function as well as cell surface protein expression,
which indicated that the aromatic group at F51 is important
for proper protein folding.
In the case of H55 and Q62, conservative replacement even
reduced uptake function of the mutants, suggesting side chain
structures of these residues are irreplaceable for the uptake
function. Alanine replacement of L58, Q62, and S66 and valine
substitution of A61 significantly decreased the binding affinity
for ES, indicating that these residues may be involved in
substrate binding. All mutants except L58A showed signifi-
cantly reduced $V_{\text{max}}$, which suggested that these amino acid
residues are crucial for substrates turnover by OATP2B1 as
well. These results indicated that L58, A61, Q62, and S66 may
be part of the substrate binding site and/or translocation
pathway.
In the present study, nine critical amino acids were
identified within transmembrane domain 1 of OATP2B1.
Most of these residues are important for substrate interaction,
whereas F51 is crucial for correct folding of the transporter
protein. Although so far there has not been any report relating
biologic significance to nonsynonymous genetic polymor-
phisms in OATP2B1 TMs, our search in the National Center
for Biotechnology Information database found reports of mis-
sense residues within different TMs of the transporter; in
particular, a V52I mutant with a minor allele frequency of
0.0002 was observed in OATP2B1. Based on our current study, such a mutation may affect uptake function of the transporter. Coordination analysis of results from biochemical studies with missense mutations reported in the National Center for Biotechnology Information database may help us better understand the therapeutic significance of OATP2B1 transmembrane domains.

Authorship Contributions
Participated in research design: Hong.
Conducted experiments: Fang, Huang, Chen, Xu, Xiang.
Performed data analysis: Huang, Hong.
Wrote or contributed to the writing of the manuscript: Hong.

References