Role of transmembrane domain 10 for the function of organic anion transporting polypeptide 1B1

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Abstract: The liver-specific organic anion transporting polypeptides OATP1B1 and OATP1B3 are highly homologous and share numerous substrates. However, at low concentrations OATP1B1 shows substrate selectivity for estrone-3-sulfate. In this study, we investigated the molecular mechanism for this substrate selectivity of OATP1B1 by constructing OATP1B1/1B3 chimeric transporters and by site-directed mutagenesis. Functional studies of chimeras showed that transmembrane domain 10 is critical for the function of OATP1B1. We further identified four amino acid residues, namely L545, F546, L550, and S554 in TM10, whose simultaneous mutation caused almost complete loss of OATP1B1-mediated estrone-3-sulfate transport. Comparison of the kinetics of estrone-3-sulfate transport confirmed a biphasic pattern for OATP1B1, but showed a monophasic pattern for the quadruple mutant L545S/F546L/L550T/S554T. This mutant also showed reduced transport for other OATP1B1 substrates such as bromosulphophthalein and [*D*-penicillamine^{2,5}]enkephalin. Helical wheel analysis and molecular modeling suggest that L545 is facing the substrate translocation pathway, whereas F546, L550, and S554 are located inside the protein. These results indicate that L545 might contribute to OATP1B1 function by interacting with substrates, whereas F546, L550, and S554 seem important for protein structure. In conclusion, our results show that TM10 is critical for the function of OATP1B1.

Keywords: OATP; estrone-3-sulfate; transmembrane domain; chimera; kinetics

Introduction

The first step of the removal of endogenous and xenobiotic substances from the portal circulation involves uptake into hepatocytes. Although many uncharged and lipophilic compounds can passively diffuse across the plasma membrane, carrier-mediated processes are critical for efficient hepatic uptake of solutes and many drugs. In humans, organic anion transporting polypeptides (OATPs, gene symbol *SLCO*) mediate the sodium-independent transport of a wide range of

amphipathic organic compounds.¹ So far 11 human OATPs have been identified.^{2,3} The polyspecific OATP1B1 and OATP1B3 are predominantly expressed at the basolateral membrane of hepatocytes.^{4–8} They share 80% amino acid sequence identity and have numerous common substrates including bile salts, unconjugated and conjugated bilirubin, bromosulphophthalein (BSP), steroid conjugates, thyroid hormones, eicosanoids, peptides, drugs, and the natural toxins phalloidin and microcystin.^{3,9} Recent findings for rat Oatp1a1 suggest that OATPs comprise 12 transmembrane domains (TMs).¹⁰

Despite their high-sequence similarity, OATP1B1 and 1B3 also exhibit substrate selectivity. OATP1B1 selectively transports prostaglandin E2¹¹ and at submicromolar concentrations estrone-3-sulfate.¹² OATP1B3 selectively transports cholecystokinin octapeptide

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(CCK-8), the anticancer drug docetaxel, and the cardiac glycosides digoxin and ouabain.^{11,13,14} In a previous study, we showed that the three amino acid residues Y537, S545, and T550 in TM10 of OATP1B3 are critical for CCK-8 transport.¹⁵ A recently published study demonstrated that TM8 and TM9 are critical for substrate recognition for OATP1B1.¹⁶ However, in this study, we extend the constructs to include TM10 and report our results that demonstrate the importance of TM10 for the function of OATP1B1.

Results

Estrone-3-sulfate transport by OATP1B1, OATP1B3, and chimeras T1-6 and T7-12

To investigate which half of OATP1B1 would be more important for estrone-3-sulfate transport, we constructed two chimeras T1-6 and T7-12 as shown in Figure 1(A). Chimera T1-6 was made of OATP1B1 residues 1–275 fused to OATP1B3 residues 276–702, whereas T7-12 consisted of OATP1B3 residues 1–275 fused to OATP1B1 residues 276–691. Both these chimeras together with full-length OATP1B1 and OATP1B3 were transiently expressed in HEK293 cells. Quantitation of surface expression after correction with the loading control Na⁺/K⁺ ATPase revealed that OATP1B1 and OATP1B3 were equally expressed at the plasma membrane of HEK293 cells. Chimera T1-6 was expressed at 51% of OATP1B1 and chimera T7-12 at 156%. Because of these unequal expression levels, all functional results were always corrected for the different expression levels of the constructs. Figure 1(B) summarizes estrone-3-sulfate transport mediated by the four constructs and shows that estrone-3-sulfate indeed was selectively transported by OATP1B1. Both OATP1B3 and chimera T1-6 did not transport estrone-3-sulfate, whereas chimera T7-12 transported estrone-3-sulfate to about 70% of OATP1B1. These results indicate that the C-terminal half of OATP1B1 contains the key molecular elements that are required for estrone-3-sulfate transport.

Chimeras derived from the C-terminal half of OATP1B1 and OATP1B3 and their function

To narrow down the critical region for OATP1B1-mediated estrone-3-sulfate transport, we constructed additional chimeras containing two (T1-8) or four (T1-10) additional TMs of OATP1B1 or two (T9-12) or four (T11-12) additional TMs of OATP1B3 [Fig. 2(A)].

Surface biotinylation and western-blot experiments showed that surface expression levels of chimeras T1-10 (68% of OATP1B1), T9-12 (118%), and T11-12 (81%) were all comparable with OATP1B1, whereas chimera T1-8 (13%) was hardly expressed at the plasma membrane [Fig. 2(B)]. The difference in the molecular weights of some of the different chimeras is most likely due to different glycosylation patterns of OATP1B1 and OATP1B3.

Figure 1. Schematic representation of human OATP1B1, OATP1B3, and chimeras T1-6 and T7-12, and their estrone-3-sulfate transport in HEK293 cells. **A:** Chimera T1-6 consisted of TMs 1–6 (residues 1–275) of OATP1B1 and TMs 7–12 (residues 276–702) of OATP1B3, whereas chimera T7-12 consisted of TMs 1–6 (residues 1–275) of OATP1B3 and TMs 7–12 (residues 276–691) of OATP1B1. **B:** Uptake of 0.1 μ M estrone-3-sulfate was measured at 37°C for 1 min with empty vector and OATP-expressing HEK293 cells. Net uptake was obtained by subtracting the uptake of empty vector transfected cells from the uptake of OATP-expressing cells. Final results were obtained by normalizing net uptake to the surface expression level of the respective proteins. Values were calculated as picomoles/(milligrams of total protein $\times$ normalization ratio)/minute, where normalization ratio = surface expression level_{mutant}/surface expression level_{OATP1B1}. Asterisks indicate a $P < 0.05$ level of significant difference from OATP1B1.

Figure 2(C) summarizes estrone-3-sulfate transport activities of the chimeric transporters together with OATP1B1 and OATP1B3 after correction for surface expression. It can clearly be seen that adding two TMs of OATP1B1 to T1-8 increased estrone-3-sulfate transport from 6% (chimera T1-8) to 65% (chimera T1-10) of OATP1B1. On the other hand, adding two or four TMs of OATP1B3 to T7-12 reduced estrone-3-

sulfate transport from 70% (chimera T7-12) to 32% (chimera T9-12) and 3% (chimera T11-12). These results demonstrated that the region of TMs 9 and 10 seems to be important for OATP1B1-mediated estrone-3-sulfate transport.

Chimeras focusing on the region of TMs 9 and 10

To further narrow down the region important for estrone-3-sulfate transport, we constructed two additional chimeras. Chimera T9 was obtained by replacing TM9 and its preceding loop of OATP1B1 with their counterparts of OATP1B3, and chimera T10 was

obtained by replacing TM10 and its preceding loop of OATP1B1 with their counterparts of OATP1B3 [Fig. 3(A)]. Chimera T9 had normal surface expression, whereas chimera T10 was not expressed on the surface at all [Fig. 3(A)]. These results indicated that either TM10 and/or extracellular loop 5 (EL5) are crucial for surface expression of OATP1B1. Therefore, we constructed two additional chimeras, EL5 and TM10 [Fig. 3(B)]. Chimera EL5 showed normal surface expression while similar to chimera T10, chimera TM10 was not expressed on cell surface [Fig. 3(B)]. This result indicated that amino acid residues in TM10 (residues 528–560) but not in EL5 are important for surface expression of OATP1B1. Given that neither T10 nor TM10 was expressed at the plasma membrane, at this step we only measured estrone-3-sulfate transport of the two normally expressed chimeras T9 and EL5. As summarized in Figure 3(C), both constructs had similar transport activities as wild-type OATP1B1, indicating that both TM 9 and extracellular loop 5 are not important for OATP1B1-mediated estrone-3-sulfate transport and therefore suggesting that amino acid residues in TM10 (residues 528–560) are critical for the function of OATP1B1.

Chimeras focusing on TM10 and identification of key residues for OATP1B1-mediated estrone-3-sulfate transport

As indicated earlier, the transport activities of chimeras T1-6, T7-12, T1-8, T9-12, T1-10, T11-12, T9, and EL5 provided indirect but strong evidence that TM10 plays a pivotal role for OATP1B1 function. Amino acid sequence comparison between OATP1B1 and OATP1B3 reveals that 14 residues are different in TM10 between

Figure 2. Schematic representation of chimeras T1-8, T9-12, T1-10, and T11-12, their surface expression and estrone-3-sulfate transport. A: Chimera T1-8 consisted of TMs 1–8 of OATP1B1 (residues 1–394) and TMs 9–12 of OATP1B3 (residues 395–702), whereas chimera T9-12 consisted of TMs 1–8 of OATP1B3 (residues 1–394) and TMs 9–12 of OATP1B1 (residues 395–691); chimera T1-10 consisted of TMs 1–10 of OATP1B1 (residues 1–560) and TMs 11–12 of OATP1B3 (residues 561–702), whereas chimera T11-12 consisted of TMs 1–10 of OATP1B3 (residues 1–560) and TMs 11–12 of OATP1B1 (residues 561–691). B: Western-blot analysis of surface biotinylated proteins detected with an anti-His antibody. The plasma membrane marker Na⁺/K⁺ ATPase α -subunit was used as protein loading control. C: Uptake of 0.1 μ M estrone-3-sulfate was measured at 37°C for 1 min with empty vector and OATP-expressing HEK293 cells. The net uptake was obtained by subtracting the uptake of empty vector transfected cells from the uptake of OATP-expressing cells. Final results were obtained by normalizing net uptakes to the surface expression levels of the respective proteins. Asterisks indicate a $P < 0.05$ level of significant difference from OATP1B1.

Figure 3. Schematic representation of chimeras T9, T10, EL5, and TM10, their surface expression and estrone-3-sulfate transport. A: Chimera T9 was obtained by replacing TM9 together with the loop preceding it (residues 395–432) in OATP1B1 with the corresponding region of OATP1B3. Chimera T10 was obtained by replacing TM10 together with the loop preceding it (residues 433–560) in OATP1B1 with the corresponding region of OATP1B3. B: Chimera EL5 was obtained by replacing extracellular loop 5 (residues 433–527) of OATP1B1 with that of OATP1B3, and TM10 was obtained by replacing TM10 (residues 528–560) of OATP1B1 with TM10 of OATP1B3. C: Uptake of 0.1 μ M estrone-3-sulfate by HEK293 cells transfected with OATP1B1, OATP1B3, chimeras T9 and EL5. Uptake was measured at 37°C for 1 min with empty vector and OATP-expressing HEK293 cells. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from the uptake of cells transfected with OATP-expressing cells. Final results were obtained by normalizing net uptake to the surface expression level of the respective proteins. Asterisks indicate a $P < 0.05$ level of significant difference from OATP1B1.

the two transporters [Fig. 4(A)]. To investigate the role of TM10 for the function of OATP1B1, we divided this TM into three parts: Part 1 comprising residues

528–537, Part 2 comprising residues 543–550, and Part 3 comprising residues 554–559. By replacing each part with its corresponding region of OATP1B3, we constructed the three chimeras P1, P2, and P3 [Fig. 4(B), left panel] that all were expressed at the cell surface [Fig. 4(B), right panel]. Then, we tested these three chimeras for their ability to transport estrone-3-sulfate. Figure 5, Panel I shows that chimera P1 had transport activity comparable with OATP1B1, whereas chimeras P2 and P3 showed significantly reduced estrone-3-sulfate transport activities (20 and 35% of OATP1B1, respectively). When we combined chimeras P2 and P3 into chimera P2_P3, it resulted in a further reduction of estrone-3-sulfate transport to only 3% of OATP1B1 (Fig. 5, Panel I), demonstrating that Parts 2 and 3 of TM10 (residues 543–559) are critical for OATP1B1-mediated estrone-3-sulfate transport with Part 2 (residues 543–550) playing a more important role than Part 3 (residues 554–559), because it showed lower transport activity.

Figure 4. Amino acid sequence alignment of TM10 of OATP1B1 with OATP1B3, and chimeras focusing on TM10. A: Sequence alignment of TM10 (residues 528–560) between OATP1B1 and OATP1B3, showing 14 amino acids different in this region (marked with “*”), namely (1B1)D528N(1B3), A529T, Y535F, F536I, F537Y, L543I, L545S, F546L, L550T, S554T, H555F, V556I, M557L, and I559T. TM10 was divided into three parts: Parts 1, 2, and 3 comprise residues 528–537, 543–550, and 554–559, respectively. B: Schematic representation of chimeras P1, P2, and P3 and their surface expression. Chimeras were constructed by replacing Parts 1, 2, and 3 with their corresponding regions of OATP1B3, respectively.

Figure 5. Uptake of estrone-3-sulfate by HEK293 cells transfected with OATP1B1, OATP1B3, and different chimeras, the quadruple, triple, and single mutants. Uptake of 0.1 μ M estrone-3-sulfate was measured at 37°C for 1 min with empty vector transfected and OATP-expressing HEK293 cells. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from the uptake of OATP-expressing cells. Final results were obtained by normalizing net uptake to the surface expression level of the respective proteins. Asterisks indicate a $P < 0.05$ level of significant difference from OATP1B1.

To identify which residues in these two parts of TM10 are important to retain OATP1B1-mediated estrone-3-sulfate transport, we characterized the following constructs. Since Part 2 seems more important than Part 3, we kept Part 2 unchanged and combined it with individual amino acid residues of Part 3, resulting in the five constructs P2_554, P2_555, P2_556, P2_557, and P2_559. Functional studies showed that these five constructs retained only 7–31% of the transport activity of OATP1B1 with construct P2_554 having the lowest activity (Fig. 5, Panel II). These results indicated that in Part 3 of TM10 S554 is the most important amino acid residue for estrone-3-sulfate transport. Because Part 2 has four amino acid residues that are different in OATP1B1 and 1B3, we then combined S554 in Part 3 with any three of the four residues that differ between OATP1B1 and OATP1B3 in Part 2 (residues L543, L545, F546, and L550) and constructed the four quadruple mutants L543I/L545S/F546L/S554T, L543I/L545S/L550T/S554T, L543I/F546L/L550T/S554T, and L545S/F546L/L550T/S554T. Similar to construct P2_554, mutant L545S/F546L/L550T/S554T had very low transport activity (10% of OATP1B1), whereas the other mutants had higher estrone-3-sulfate transport activities (28–64% of OATP1B1) (Fig. 5, Panel III), indicating that L545, F546, and L550 are the most important residues in Part 2 of TM10 for OATP1B1-mediated estrone-3-sulfate transport. To investigate whether all four residues L545, F546, L550, and S554 had to be mutated simultaneously, we constructed triple and single mutants

and tested their functions. All triple mutants showed significantly higher transport activities (25–73% of OATP1B1) than the quadruple mutant L545S/F546L/L550T/S554T (10% of OATP1B1) (Fig. 5, Panel IV) and all four single mutants L545S, F546L, L550T, and S554T retained almost normal estrone-3-sulfate transport activity (>80% of OATP1B1) (Fig. 5, Panel IV). We also constructed the single mutants for other 10 residues in TM10 and their transport activities were comparable with wild-type OATP1B1 (>70% of OATP1B1) (data not shown). Taken together, these results indicate that residues L545, F546, L550, and S554 in TM10 are crucial for OATP1B1-mediated estrone-3-sulfate transport and that simultaneous mutation of all four residues is necessary to effectively compromise OATP1B1's function.

Kinetics of estrone-3-sulfate transport mediated by OATP1B1 and the quadruple mutant

Because the quadruple mutant L545S/F546L/L550T/S554T showed dramatically reduced estrone-3-sulfate transport, we performed kinetics of OATP1B1- and quadruple mutant-mediated estrone-3-sulfate transport and compared their kinetic parameters. The Eadie–Hofstee plot in Figure 6(A) confirmed earlier observations that transport of estrone-3-sulfate by OATP1B1 is biphasic and consists of a high-affinity low-capacity and a low-affinity high-capacity component.^{17,18} The K_m and V_{max} values for the high- and low-affinity components are summarized in Table I and were 0.22 ± 0.04 and 312 ± 17 μ M, and 25.0 ± 1.2 and 298 ± 6 pmol (normalized mg)^{−1} min^{−1}, respectively. The quadruple mutant, however, only showed a single high-affinity low-capacity component for estrone-3-sulfate [Fig. 6(B)]. The respective K_m and V_{max} values were 2.5 ± 0.7 μ M and 23.5 ± 2.4 pmol (normalized mg)^{−1} min^{−1} (Table I).

Discussion

In this study, we have investigated the molecular mechanism for the substrate selectivity of OATP1B1 by constructing and functionally studying a series of OATP1B1/1B3 chimeric transporters and by site-directed mutagenesis. Our results demonstrated that similar to our previous findings for OATP1B3,¹⁵ TM10 which is located in the C-terminal half of OATP1B1 is critical for the protein's function. We further identified four amino acid residues L545, F546, L550, and S554 in TM10 to be important for estrone-3-sulfate transport by OATP1B1. The N-terminal half of OATP1B1 has little effect on the protein's function.

TM10 has before been suggested to be important for OATP-mediated transport. Besides our results with OATP1B3,¹⁵ cysteine-scanning mutagenesis experiments of rat Oatp2b1 indicated that TM10 is part of the substrate binding site.¹⁹ Thus, TM10 seems to be important not only in the OATP1 subfamily but also in the OATP2 subfamily. A recent study showed that TMs

Figure 6. Eadie-Hofstee plots of estrone-3-sulfate uptake by (A) OATP1B1 and (B) the quadruple mutant L545S/F546L/L550T/S554T. Uptake of estrone-3-sulfate was measured at various concentrations from 0.02 to 500 μM for OATP1B1 and from 0.1 to 10 μM for quadruple mutant L545S/F546L/L550T/S554T. Uptake was conducted at 37°C for 1 min with empty vector and OATP-expressing HEK293 cells. The net uptake was obtained by subtracting the uptake of cells transfected with empty vector from the uptake of OATP-expressing cells. Final results were obtained by normalizing net uptake to the surface expression levels of the respective proteins. Lines represent the calculated values using the kinetic parameters obtained by nonlinear regression analysis.

8 and 9 are also involved in OATP1B1-mediated estrone-3-sulfate transport.¹⁶ Although our results support those findings, the role of TMs 8 and 9 for the function of OATP1B1 does not seem to be as critical as the role of TM10: chimera T8 (residues 359–394 of OATP1B1 being replaced by the corresponding residues of OATP1B3) retained 32% activity of OATP1B1 (data not shown) and chimera T9 (residues 395–432 of OATP1B1 being replaced by the corresponding residues of OATP1B3) retained 94% activity of OATP1B1 [Fig. 3(C)], whereas chimera P2_P3 (residues 543–559 of OATP1B1 being replaced by the corresponding residues of OATP1B3) only retained 3% activity of OATP1B1 (Fig. 5, Panel I). Unfortunately, Miyagawa et al.¹⁶ did not give detailed information on the exact amino acids

that were replaced in their study and did not correct their transport results for protein expressed at the plasma membrane, which makes a direct comparison difficult.

Numerous polymorphisms have been reported for OATP1B1.^{3,20} However, the only polymorphism in TM10 known so far is L543W and it was found in a Japanese patient who experienced pravastatin-induced myopathy.²¹ Mutation at position 543 in our study did not have a big impact on the function of OATP1B1, but the leucine normally present at this position was replaced by the isoleucine found in OATP1B3, which very likely did not have the same impact on the three-dimensional structure of OATP1B1 as a change to the bulky tryptophan. Furthermore, given that only the quadruple mutant showed such a dramatic loss of function and the different triple mutants retained 25–73% of transport function, it is very unlikely that a single polymorphism at any of the four amino acids would result in a profound decrease of OATP1B1 function.

OATP1B1 shares numerous substrates like BSP or [D-penicillamine^{2,5}]enkephalin (DPDPE) with other OATPs.⁹ Therefore, we tested whether the quadruple mutant would retain normal BSP and DPDPE transport. It turned out that transport of BSP was drastically reduced [Fig. 7(A)] and uptake of DPDPE was essentially absent for the quadruple mutant [Fig. 7(B)] when compared with wild-type OATP1B1. Thus, the quadruple mutation affected the transport function of OATP1B1 not only for estrone-3-sulfate, but also for other substrates such as BSP and DPDPE. This is different from our findings with OATP1B3 where chimera 1B3_TM10 only impaired transport of CCK-8, whereas uptake of DPDPE and taurocholate was normal or even increased.¹⁵

Helical wheel analysis of TM10 revealed that residue L545 is located on one side of TM10, whereas residues F546, L550, and S554 are located on the other side of the helix (Fig. 8). According to our model for OATP1B3,¹⁵ residue L545 faces the substrate translocation pathway, whereas residues F546, L550, and S554 face inside the helical bundle. Thus, only L545 of the quadruple mutant might interact with the substrate directly. The other three residues (F546, L550, and S554) are located inside the protein and should not

Table I. Kinetic Parameters of Estrone-3-Sulfate Transport Mediated by OATP1B1 and the Quadruple Mutant L545S/F546L/L550T/S554T

	K_m (μM)	$V_{\max}$ [pmol (normalized mg) ⁻¹ min ⁻¹]	$V_{\max}/K_m$
OATP1B1	0.22 ± 0.04	25.0 ± 1.2	113.6
	312 ± 17	298 ± 6	0.96
Quadruple mutant L545S/F546L/L550T/S554T	2.5 ± 0.7	23.5 ± 2.4	9.4

Figure 7. Uptake of (A) BSP and (B) DPDPE by HEK293 cells transfected with OATP1B1 and the quadruple mutant L545S/F546L/L550T/S554T. Uptake of hot BSP and DPDPE were measured at 37°C for 1 min with empty vector transfected and OATP-expressing HEK293 cells. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from the uptake of OATP-expressing cells. Final results were obtained by normalizing net uptake to the surface expression level of the respective proteins. Asterisks indicate a $P < 0.05$ level of significant difference from OATP1B1.

contribute to the formation of the substrate translocation pathway. Therefore, we suggest that residues F546, L550, and S554 affect the function of OATP1B1 by affecting its overall structure, which could explain why the effect of the quadruple mutant is not restricted to estrone-3-sulfate.

Several studies have shown that OATP1B1-mediated substrate transport most likely occurs via different transport/binding sites. Two studies demonstrated biphasic saturation kinetics with a high-affinity low-capacity and a low-affinity high-capacity component for OATP1B1-mediated estrone-3-sulfate transport.^{17,18} We could confirm these studies and also demonstrate that uptake of estrone-3-sulfate by OATP1B1 exhibited a biphasic kinetics with high-affinity low-capacity and low-affinity high-capacity components having K_m values of 0.22 and 312 μM and V_{max} values of 25.0 and

298 pmol (normalized mg)⁻¹ min⁻¹, respectively (Fig. 6 and Table I). By contrast, estrone-3-sulfate transport by the quadruple mutant exhibited only a single rather high-affinity low-capacity component with a K_m values of 2.5 μM and a V_{max} value of 23.5 pmol (normalized mg)⁻¹ min⁻¹. Thus, the quadruple mutant has lost the low-affinity high-capacity component of OATP1B1. Comparison of the kinetic constants shows that the apparent binding affinity of the quadruple mutant for estrone-3-sulfate (K_m of 2.5 μM) decreased 10-fold with respect to the high-affinity component of wild-type OATP1B1 (K_m of 0.22 μM) and that their transport capacities were comparable [V_{max} of 23.5 and 25.0 pmol (normalized mg)⁻¹ min⁻¹, respectively]. This suggests that at low substrate concentrations, the reduced transport of estrone-3-sulfate by the quadruple mutant is most likely due to the reduced binding affinity but not due to a changed transport capacity. In addition, the loss of one component of OATP1B1 in the quadruple mutant provides further evidence that these mutations cause structural alterations in this transport protein.

In conclusion, in this study, we have identified that TM10 is the molecular element critical for the function of OATP1B1. Four amino acid residues within TM10, namely L545, F546, L550, and S554, are important for normal transport of OATP1B1. However, simultaneous mutation of these four residues is required to impair OATP1B1-mediated transport. Helical wheel analysis and molecular modeling indicate that L545 faces the putative substrate translocation pathway and

Figure 8. Helical wheel analysis of residues 537–554 in TM10 of OATP1B1. Residues 537–554 of OATP1B1 are shown in a helical wheel projection created with the helical wheel applet written by Edward K. O'Neil and Charles M. Grisham (<http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html>). Polar residues are shown in black and hydrophobic residues are in gray. Residue L545 which is putatively facing the substrate translocation pathway, is indicated within ellipses; residues F546, L550, and S554, which are located at the opposite side of the helix, are boxed.

can interact directly with substrates, whereas F546, L550, and S554 are predicted to face the inside of the protein. These three amino acid residues most likely affect normal protein structure.

Materials and Methods

Chemicals and reagents

Radiolabeled [³H]estrone-3-sulfate (57.3 Ci/mmol) was purchased from PerkinElmer (Waltham, MA) and unlabeled estrone-3-sulfate was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was obtained from Hyclone (Logan, UT) while all other cell culture reagents, Lipofectamine 2000 and the vector pcDNA5/FRT were from Invitrogen (Carlsbad, CA). The QuikChange kit for site-directed mutagenesis was from Stratagene (La Jolla, CA). Sulfo-*N*-hydroxysuccinimide-SS-biotin, streptavidin-agarose beads, and the BCA protein assay kit were purchased from Pierce Chemical (Rockford, IL). The polyclonal anti-His antibody was from Covance (Princeton, NJ) whereas the monoclonal antibody to detect Na⁺/K⁺-ATPase α -subunit was purchased from Abcam (Boston, MA). Poly-D-lysine was from Sigma-Aldrich (St. Louis, MO) and the protease inhibitor cocktail (Complete, Mini, EDTA-free) was from Roche (Indianapolis, IN).

Construction of chimeric transporters and mutants

To use a single antibody to detect all constructs, we inserted a six His-tag at the C-terminal ends of the open reading frames of human OATP1B1*1b²² and OATP1B3 haplotype 1²³ by PCR. The resulting constructs were cloned into the vector pcDNA5/FRT (Invitrogen, Carlsbad, CA) via *NheI* and *NotI* sites. For the construction of chimeric transporters between OATP1B1 and OATP1B3, the junction sites were determined based on the predicted topology of OATP1B1 and OATP1B3 by the software TMPred (http://www.ch.embnet.org/software/TMPRED_form.html), and an overlapping PCR strategy was employed with overlapping regions of 20–23 bases. Site-directed mutagenesis was performed using the QuikChange kit and all constructs were confirmed by DNA sequencing.

Protein expression in HEK293 cells

Human embryonic kidney (HEK293) cells were grown at 37°C as described in Ref. 15. Plasmids containing the cDNAs of OATP1B1, OATP1B3, and chimeras or mutants were transiently transfected into HEK293 cells using Lipofectamine 2000 following the manufacturer's instruction. After 48 h at 37°C, the cells were used for protein surface expression and transport assays.

Cell surface biotinylation and immunoblot analysis

HEK293 cells were grown in six-well plates coated with poly-D-lysine and transfected as described earlier. After 48 h, cells were washed twice with 2 mL of ice-cold phosphate-buffered saline (PBS) and then treated for 1 h at 4°C with 1 mL of sulfo-*N*-hydroxysuccinimide-SS-biotin (1 mg/mL in PBS). After this, cells were washed three times with 2 mL of ice-cold PBS containing 100 mM glycine and incubated for 10 min at 4°C in the same buffer. Then, cells were lysed with 700 μ L of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4, containing protease inhibitors) for 1 h at 4°C with shaking. The resulting lysates were centrifuged for 2 min at 10,000g and the supernatants were incubated with 140 μ L of streptavidin-agarose beads for 1 h at room temperature under constant agitation. The beads were then centrifuged at 850g for 1 min, washed three times with ice-cold lysis buffer, and incubated with 150 μ L of 2 $\times$ Laemmli buffer containing 100 mM dithiothreitol at room temperature for 30 min to recover the cell surface proteins. Samples were separated using SDS-polyacrylamide gel electrophoresis followed by western-blot analysis. All constructs and mutant proteins were detected using a polyclonal anti-His antibody (1:2500 dilution), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution). Na⁺/K⁺ ATPase was used as loading control for normalization and was detected with a mouse anti-Na⁺/K⁺ ATPase α subunit antibody (1:5000 dilution). ECL plus (Amersham Biosciences, Piscataway, NJ) was used for detection. Protein band intensities were quantified with the Quantity One analysis software (Bio-Rad Laboratories, Hercules, CA).

Functional studies of transporters in HEK293 cells

For functional studies HEK293 cells were grown in 12-well plates and uptake was measured 48 h after transfection. Cells were washed with 2 mL of prewarmed uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, pH adjusted to 7.4 with Trizma base) for three times. Then, uptake was started by adding 400 μ L of uptake buffer containing radiolabeled substrate. After 1 min of incubation, uptake was stopped by removing the uptake solution and washing the cells four times with 2 mL of ice-cold uptake buffer. Then, the cells were solubilized with 500 μ L of 1% Triton X-100 and 300 μ L were used for liquid scintillation counting. From the remaining, lysate protein concentration was determined for each well using the BCA protein assay kit. Cells transfected with the empty vector served as background control in all experiments. Initial tests demonstrated that uptake was linear over at least 1 min at substrate concentrations of 0.1 and 100 μ M. Therefore, all uptake and kinetic

experiments were done under these initial linear conditions. To calculate transporter-specific uptake, we subtracted the background uptake from OATP-transfected uptake and normalized it with protein surface expression levels.

Data analysis

Uptake experiments were performed in duplicate and repeated at least once. Data with error bars represent the mean $\pm$ standard deviation. To analyze whether the groups were different from the control, one-way analysis of variance (ANOVA) was performed followed by the Bonferroni *t*-test with SigmaStat 3.5 (Systat Software, San Jose, CA). The *P* value for statistical significance was set to be <0.05 . The kinetic parameters were obtained by nonlinear regression fitting with the Enzyme Kinetics Module of SigmaPlot (Systat Software, San Jose, CA).

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