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Several Conserved Positively Charged Amino Acids in OATP1B1 are Involved in Binding or Translocation of Different Substrates

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Abstract OATP1B1 and 1B3 are related transporters mediating uptake of numerous compounds into hepatocytes. A putative model of OATP1B3 with a "positive binding pocket" containing conserved positively charged amino acids was predicted (Meier-Abt et al. J Membr Biol 208: 213–227, 2005). Based on this model, we tested the hypothesis that these positive amino acids are important for OATP1B1 function. We made mutants and measured surface expression and uptake of estradiol-17β-glucuronide, estrone-3-sulfate and bromosulfophthalein in HEK293 cells. Two of the mutants had low surface expression levels: R181K at 10% and R580A at 30% of wild-type OATP1B1. A lysine at position 580 (R580K) rescued the expression of R580A. Mutations of several amino acids resulted in substrate-dependent effects. The largest changes were seen for estradiol-17β-glucuronide, while estrone-3-sulfate and bromosulfophthalein transport were less affected. The wild-type OATP1B1 K_m value for estradiol-17β-glucuronide of 5.35 $\pm$ 0.54 μM was increased by R57A to 30.5 $\pm$ 3.64 μM and decreased by R580K to 0.52 $\pm$ 0.18 μM. For estrone-3-sulfate the wild-type high-affinity K_m value of 0.55 $\pm$ 0.12 μM was increased by K361R to 1.8 $\pm$ 0.47 μM and decreased by R580K to 0.1 $\pm$ 0.04 μM. In addition, R580K reduced the V_{max} values for all three substrates to <25% of wild-type OATP1B1. Mutations at intracellular K90, H92 and R93 mainly affected V_{max} values for estradiol-17β-glucuronide uptake. In conclusion, the conserved amino acids R57, K361 and R580 seem to be part of the substrate binding sites and/or translocation pathways in OATP1B1.

Keywords Organic anion transporting polypeptide · Liver · Transporter · Site-directed mutagenesis

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Introduction

OATP1B1 is a transport protein expressed at the basolateral membrane of human hepatocytes (Abe et al. 1999, 2001; Hsiang et al. 1999; König et al. 2000). It belongs to the organic anion transporting polypeptide superfamily (OATP, gene symbol *SLCO*) (Hagenbuch and Meier 2004) and mediates the sodium-independent transport of a wide range of structurally unrelated amphipathic organic compounds (Hagenbuch and Gui 2008; König et al. 2006). Due to its broad substrate spectrum, OATP1B1 is important for hepatic clearance of many drugs and other xenobiotics. In order to explain and prevent potential adverse drug interactions that occur at the transporter level, a thorough understanding of the transport mechanisms of OATP1B1 is essential.

Several functional studies have suggested that there are multiple substrate recognition sites or translocation pathways for the different OATP1B1 substrates (Gui et al. 2008; Noe et al. 2007; Tamai et al. 2001). Two recent studies identified transmembrane domains (TMs) 8 and 9 (Miyagawa et al. 2009) and 10 (Gui and Hagenbuch 2009) to be important for OATP1B1-mediated substrate transport. Furthermore, several amino acids within TM10 were identified to be important for substrate binding (L545) or protein structure/folding (F546, L550, S554) (Gui and Hagenbuch 2009).

Because so far none of the OATPs has been crystallized, comparative modeling has been used to predict the structure of OATPs. Based on their 12-transmembrane domain topology (Wang et al. 2008) common to members of the major facilitator superfamily (MFS), the known crystal structures of two bacterial transporters in the MFS were used as templates to generate a 3D structural model for OATP1B3 (Meier-Abt et al. 2005). Based on this model, Meier-Abt et al. (2005) proposed a putative binding pocket within an aqueous pore that contains several positively charged amino acid residues conserved throughout the OATP1 family. In a recent study with OATP1B3, K41 in TM1 and R580 in TM11 were confirmed to be important for bromosulfophthalein (BSP) transport (Glaeser et al. 2010). However, the roles of the positively charged amino acids within the putative binding pocket for OATP1B1-mediated transport are still unknown. Therefore, in this study, we performed site-directed mutagenesis of these conserved positively charged amino acids and characterized the mutant proteins with respect to surface expression and transport function. We determined the significance of the extracellular R57 and K361, of the transmembrane R181 (TM4) and R580 (TM11) and of the intracellular K90, H92 and R93 for OATP1B1-mediated transport of the model substrates estradiol-17β-glucuronide, estrone-3-sulfate and BSP.

Materials and Methods

Materials

Radiolabeled [^{3}H]-estradiol-17β-glucuronide (46.9 Ci/mmol) and [^{3}H]-estrone-3-sulfate (57.3 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). Radiolabeled [^{3}H]-sulfobromophthalein (14.5 Ci/mmol) was purchased from International Isotope Clearing House (Leawood, KS). Unlabeled chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The anti-OATP1B1 K23 antibody was kindly provided by Dr. Bruno Stieger (University Hospital, Zurich, Switzerland).

Site-Directed Mutagenesis

Human OATP1B1*1b was subcloned into the pcDNA5/FRT vector (Invitrogen, Carlsbad, CA) and is considered wild-type compared to the introduced mutations. Single-amino acid mutations were introduced by site-directed mutagenesis using the QuickChange® system (Stratagene, La Jolla, CA), following the manufacturer's instructions. Primers used in the mutagenesis reactions are listed in Table 1. Plasmid DNA was prepared using HiSpeed Plasmid Midi or Maxi Kits (Qiagen, Germantown, MD), and both strands of all constructs were sequenced to confirm the presence of the designed mutations and the absence of additional spontaneous mutations.

Protein Expression in HEK293 Cells

Human embryonic kidney (HEK293) cells were grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified eagle medium high glucose (Invitrogen), supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Twenty-four hours before transfection, HEK293 cells were harvested by trypsinization and replated at 250,000 cells/well in 24-well plates (coated with 0.1 mg/ml poly-D-lysine). The transfection mixture consisted of 0.8 µg of plasmid DNA and 2 µl Lipofectamine™ 2000 (Invitrogen). Transfected cells were incubated at 37°C for 48 h before use.

Surface Biotinylation

The method described here is a modification of the one published by Ho et al. (2004). Briefly, 48 h after transfection, HEK293 cells were washed with ice-cold phosphate-buffered saline Ca^{2+}/Mg^{2+} (PBS-CM; 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4) and then treated with 1 mg/ml membrane-impermeable biotinylating agent sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce, Rockford, IL) at 4°C for 1 h. Subsequently, cells were washed three times with ice cold PBS-CM containing 100 mM glycine to remove the remaining labeling reagent. Then, cells were lysed in 300 µl lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS, pH 7.5) containing protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN) at 4°C with constant agitation for 1 h. Following centrifugation at 10,000×g (4°C) for 2 min, 50 µl of NeutrAvidin (Pierce) beads were added to 250 µl of cell lysate supernatant and incubated at room temperature for 1 h with constant agitation. Beads were then washed four times with lysis buffer, and the biotinylated proteins were recovered from the beads by incubating with 2× Laemmli buffer with 50 mM DTT for 30 min at room temperature.

SDS-PAGE and Western Blotting

Forty microliters of surface proteins were separated on 8% SDS-PAGE minigels at 150 V for 1 h and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h with 5% nonfat dry milk in PBS at room temperature, followed by overnight

Table 1 Primers for site-directed mutagenesis

Mutation	Primers (5′–3′)
R181A	Forward: GTGTTCATGGGTAATATGCTT**GCT**GGAATAGGGGAGACTCCC
	Reverse: GGGAGTCTCCCCTATTCC**AGC**AAGCATATTACCCATGAACAC
R181K	Forward: GTGTTCATGGGTAATATGCTT**AAA**GGAATAGGGGAGACTCCCATAG
	Reverse: CTATGGGAGTCTCCCCTATTCC**TTT**AAGCATATTACCCATGAACAC
R181H	Forward: CATGGGTAATATGCTT**CAT**GGAATAGGGGAGACTCCC
	Reverse: GGGAGTCTCCCCTATTCC**ATG**AAGCATATTACCCATG
R580A	Forward: CCACTCAATGGTTATA**GCA**GCACTAGGAGGAATTCTAGC
	Reverse: GCTAGAATTCCTCCTAGTGC**TGC**TATAACCATTGAGTGG
R580K	Forward: CCACTCAATGGTTATA**AAA**GCACTAGGAGGAATTCTAGCTCC
	Reverse: GGAGCTAGAATTCCTCCTAGTGC**TTT**TATAACCATTGAGTGG
R580H	Forward: CCACTCAATGGTTATA**CAT**GCACTAGGAGGAATTCTAGCTCC
	Reverse: GGAGCTAGAATTCCTCCTAGTGC**ATG**TATAACCATTGAGTGG
R57A	Forward: GTTCCATCATTCATATAGAA**GCG**AGATTTGAGATATCCTCTTCTCTTG
	Reverse: CAAGAGAAGAGGATATCTCAAATCT**CGC**TTCTATATGAATGATGGAAC
R57K	Forward: GAA**AAG**AGATTTGAGATATCCTCTTCTC
	Reverse: AAATCT**CTT**TTCTATATGAATGATGGAAC
K361A	Forward: GGTGCTTTTACTTATGTCTTC**GCA**TACGTAGAGCAACAGTATGG
	Reverse: CCATACTGTTGCTCTACGTA**TGC**GAAGACATAAGTAAAAGCACC
K361R	Forward: TTC**AGA**TACGTAGAGCAACAGTATGG
	Reverse: GTA**TCT**GAAGACATAAGTAAAAGCACC
K90A	Forward: GTGAGTTACTTTGGATCC**GCA**CTACATAGACCAAAGTTAATTGG
	Reverse: CCAATTAACTTTGGTCTATGTAG**TGC**GGATCCAAAGTAACTCAC
K90R	Forward: GTGAGTTACTTTGGATCC**CGA**CTACATAGACCAAAGTTAATTGG
	Reverse: CCAATTAACTTTGGTCTATGTAG**TCG**GGATCCAAAGTAACTCAC
H92A	Forward: GTTACTTTGGATCCAAACTA**GCT**AGACCAAAGTTAATTGGAATCGG
	Reverse: CCGATTCCAATTAACTTTGGTCT**AGC**TAGTTTGGATCCAAAGTAAC
H92K	Forward: GAGTTACTTTGGATCCAAACTA**AAG**AGACCAAAGTTAATTGGAATCGG
	Reverse: CCGATTCCAATTAACTTTGGTCT**CTT**TAGTTTGGATCCAAAGTAACTC
H92R	Forward: CTTTGGATCCAAACTA**CGT**AGACCAAAGTTAATTGGAATCGG
	Reverse: CCGATTCCAATTAACTTTGGTCT**ACG**TAGTTTGGATCCAAAG
R93A	Forward: CTTTGGATCCAAACTACAT**GCA**CCAAAGTTAATTGGAATCGGTTG
	Reverse: CAACCGATTCCAATTAACTTTGG**TGC**ATGTAGTTTGGATCCAAAG
R93K	Forward: CTTTGGATCCAAACTACAT**AAA**CCAAAGTTAATTGGAATCGGTTG
	Reverse: CAACCGATTCCAATTAACTTTGG**TTT**ATGTAGTTTGGATCCAAAG

incubation with anti-OATP1B1 antibody (1:2,500 dilution) at 4°C. After washing with 0.1% PBS/Tween-20, the membrane was incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (Pierce) for 1 h at room temperature in 2.5% milk in PBS (1:10,000 dilution). Following extensive washing with 0.1% PBS/Tween-20, the secondary antibody was detected using the ECL kit (Amersham, Aylesbury, UK). Protein loading was normalized using the plasma membrane marker Na^+/K^+-ATPase α-subunit antibody (Abcam, Cambridge, MA; 1:5,000 dilution). Horseradish peroxidase–conjugated goat anti-mouse IgG (Pierce) was used as secondary antibody (1:10,000 dilution). Immunoblots were developed using the ECL plus kit. The intensity of protein bands was quantified using ImageJ (http://rsbweb.nih.gov/ij/). Band intensities at five or six different exposure times were quantified to ensure that the signal was within the linear range of the film.

Transport Assay

After washing the cells three times with prewarmed (37°C) uptake buffer modified from Cui et al. (2001) (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.4), 200 µl uptake buffer containing radiolabeled substrates with sufficient unlabeled compound to achieve the indicated concentrations were added to initiate transport.

After incubating for the indicated time periods, transport was terminated by four washes with ice-cold uptake buffer. Cells were lysed with 300 µl 1% Triton X-100 at room temperature for 30 min. Two hundred microliters of cell lysate were transferred to 24-well scintillation plates (Perkin Elmer, Shelton, CT), and radioactivity was measured after adding Optiphase Supermix scintillation cocktail (Perkin Elmer) in a MicroBeta liquid scintillation counter. The remaining 100 µl cell lysate were used to determine the protein concentration using the BCA™ Protein Assay (Pierce). All transport activities in transiently transfected HEK293 cells expressing wild-type or mutated OATP1B1 were measured within the initial linear time range and corrected by total protein concentration.

Kinetic Analysis

Kinetics for wild-type OATP1B1 and mutants were determined within the initial linear time range. Transport of radiolabeled estradiol-17β-glucuronide was measured from 1 to 50 µM for 1 min, transport of radiolabeled estrone-3-sulfate was measured from 0.1 to 2 µM for 30 s and transport of radiolabeled BSP was measured from 0.05 to 3 µM for 1 min. Transporter-specific uptake was obtained by subtracting the uptake into empty vector–transfected cells from the uptake into OATP1B1-transfected cells. Michaelis-Menten nonlinear curve fitting was carried out to obtain estimates of the maximal uptake rate (V_{max}) and the apparent affinity constant (K_m) (Graphpad Prism; GraphPad Software, La Jolla, CA).

Statistical Analysis

Statistical significance was calculated using two-tailed unpaired Student's t-test and $P < 0.05$ was considered significant.

Results and Discussion

Functional Characterizations of Wild-Type OATP1B1 Transiently Transfected in HEK293 Cells

Because OATP1B1 is a multispecific transporter (Hagenbuch and Gui 2008) and because for certain substrates multiple substrate binding sites have been suggested (Hagenbuch and Gui 2008; Noe et al. 2007; Tamai et al. 2001), we established normal OATP1B1 function by characterizing uptake of the three model substrates [^{3}H]-estradiol-17β-glucuronide, [^{3}H]-estrone-3-sulfate and BSP in transiently transfected HEK293 cells. OATP1B1-mediated uptake of estradiol-17β-glucuronide was linear at both low (1 µM) and high (50 µM) substrate concentrations for at least 1 min. Kinetic experiments performed at 1 min revealed a K_m value of 5.35 ± 0.54 µM, a value well within the range of published values for estradiol-17β-glucuronide reported with other expression systems (Cui et al. 2001; Gui et al. 2008; Hirano et al. 2004; König et al. 2000; Tamai et al. 2001). Similar to estradiol-17β-glucuronide, transport of estrone-3-sulfate by HEK293 cells transiently transfected with wild-type OATP1B1 was linear at least over 30 s at 0.1, 1 and 50 µM; therefore, kinetics were performed at 30 s. Although two binding sites were identified for OATP1B1-mediated estrone-3-sulfate transport (Gui and Hagenbuch 2009; Noe et al. 2007; Tamai et al. 2001), we investigated only the high-affinity site and could confirm that the K_m of 0.55 ± 0.12 µM was comparable to previously published values (Gui and Hagenbuch 2009; Hirano et al. 2004; Noe et al. 2007). Uptake of the other high-affinity substrate of OATP1B1, BSP (Cui et al. 2001; Kullak-Ublick et al. 2001) was linear over at least 1 min both at low (0.02 µM) and high (3 µM) concentrations. Therefore, concentration-dependent uptake of BSP was measured at 1 min, and the K_m value of 0.46 ± 0.04 µM was in the same range as values previously published (Cui et al. 2001; Kullak-Ublick et al. 2001). Taken together, these results demonstrated that our transient expression system with HEK293 cells was suitable to characterize uptake mediated by OATP1B1 and its mutants.

Expression of OATP1B1 Mutants in HEK293 Cells

To determine the functional effects of the individual conserved positively charged amino acids facing the putative binding pocket (Meier-Abt et al. 2005), we performed site-directed mutagenesis and changed amino acid residues at the seven positions indicated in Fig. 1; R57 and K361 at the predicted extracellular side, R181 and R580 in predicted TM 4 and 11 and K90, H92 and R93 at the predicted intracellular side of OATP1B1 were individually replaced with alanine and other charged amino acids such as lysine, arginine or histidine. Both wild-type and mutated OATP1B1 were then transiently expressed in HEK293 cells. Membrane proteins were purified using surface biotinylation, and Western blot analysis was performed using an anti-OATP1B1 antibody targeted to the cytoplasmic C-terminal end. Thus, none of these mutations would affect the antibody recognition site, and differences on the Western blots would reflect different amounts of OATP1B1 at the plasma membrane of HEK293 cells. Na^+/K^+-ATPase, a membrane protein naturally expressed in all HEK293 cells, was used as loading control for surface proteins. As demonstrated in Fig. 2a, all OATP1B1 constructs were detectable at the cell surface, two of them (R181K and R580A) at strongly reduced levels. We

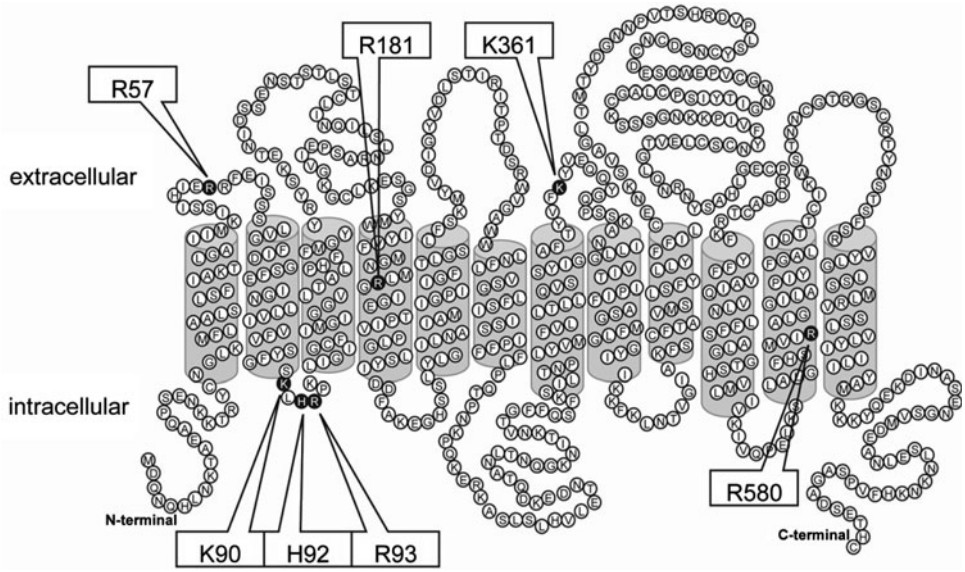

Fig. 1 Predicted topological model of human OATP1B1 with mutation sites. The topological structure of OATP1B1 was predicted by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). Amino acids mutated in this study are indicated in black, and the positions are listed in the boxes pointing to these residues

quantified Western blots from three such individual and independent experiments, and the data are shown in Fig. 2b. It can be seen that R181K and R580A were expressed at only 10 and 30% of the value of wild-type OATP1B1 at the cell surface, respectively. Total protein expression of these two mutants compared to wild-type OATP1B1 was also very low (data not shown), suggesting that there were problems with protein synthesis and/or folding rather than only problems with the translocation to the plasma membrane. Surface expression levels of R181A, R181H, R57A, H92A, H92K and H92R were between 60% and 80% of the surface expression of wild-type OATP1B1. Expression of all the other mutants at the surface of HEK293 cells was comparable to wild-type OATP1B1. None of the mutations affected HEK293 cell growth rate; therefore, the low surface expression can be explained by impaired protein synthesis, folding, stability or trafficking to the cell surface. Because the reduced surface expression of R57A and R580A could be rescued by reintroducing a positive R57K and R580K, we conclude that a positive charge at positions 57 and 580 could be important for normal synthesis and trafficking of OATP1B1 to the cell surface or for its normal anchoring in the cell membrane. Surface expression levels of R181A and R181H were 77% and 59% of wild-type OATP1B1, respectively. However, R181K surface expression was only 10% of wild-type, and the total expression level of this mutation was also drastically reduced (data not shown), indicating that a lysine at this position is not compatible with normal protein expression. This conclusion is supported by amino acid sequence alignments with over 50 OATPs/Oatps that demonstrate that at this position an arginine is absolutely conserved in the OATP1 family. OATPs/Oatps in other families have different amino acids at this position, including alanines or histidine; but none of the amino acid residues is a lysine. Thus, we conclude that a lysine at position 181, although it is positively charged like the normally found arginine, does not lead to normal surface expression of the protein. Expression levels of all mutants except for the very weakly expressed R181K and R580A correlated well with the different exposure times of the films. Therefore, the surface-corrected V_{max} values for R580A might be overcorrected.

Initial Uptake Rates of OATP1B1 Mutants

In order to screen all 16 mutants for their effects on transport of the three OATP-model substrates, we measured uptake of 1 μM estradiol-17β-glucuronide, 0.1 μM estrone-3-sulfate and 0.05 μM BSP under initial linear rate conditions. The results are summarized in Fig. 3. The extracellular R57 when mutated to an alanine or lysine exhibited substrate-specific effects, with uptake of estradiol-17β-glucuronide being the most and estrone-3-sulfate the least compromised. BSP-uptake rates were between the rates of estradiol-17β-glucuronide and estrone-3-sulfate (Fig. 3a). The other extracellular residue, K361, showed less of a substrate-specific effect. In particular, K361R exhibited only about 50% of the uptake rates for all three substrates compared to wild-type OATP1B1 (Fig. 3a). For the two transmembrane residues, uptake rates for estradiol-17β-glucuronide and estrone-3-sulfate were in general lower than the ones for BSP (Fig. 3b), except for R181A and R580H which had similar rates for all three different substrates. For the intracellular residues (Fig. 3c) mutations at K90 had no effect on any of the three substrates tested. At H92 all mutations resulted in a stronger decrease of estradiol-17β-glucuronide transport rates compared to

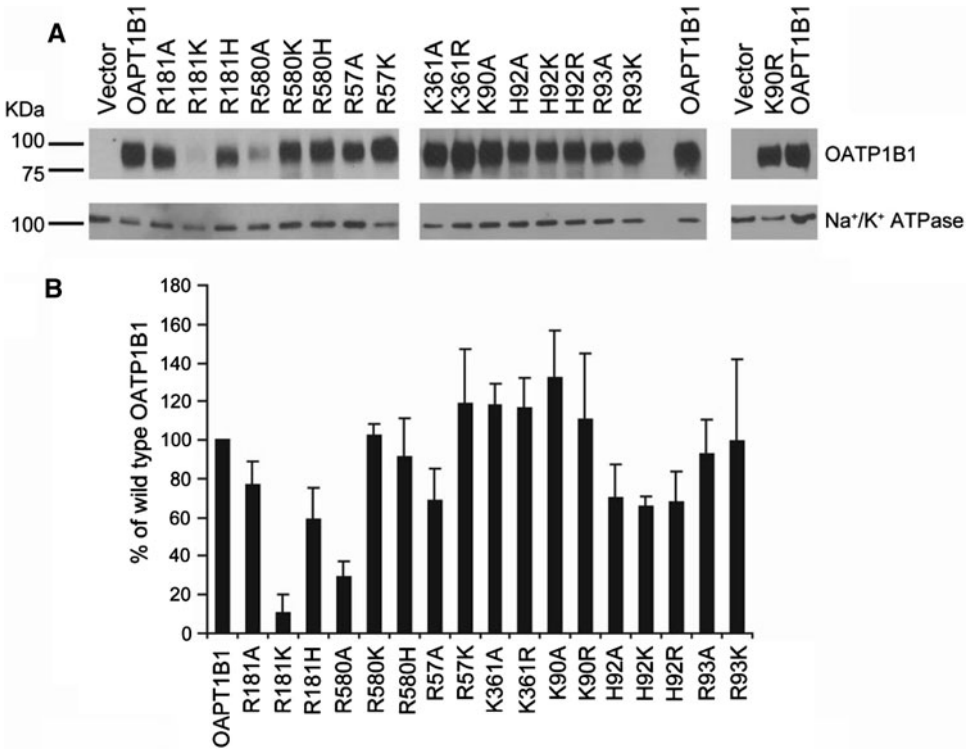

Fig. 2 Surface expression of OATP1B1 mutants in HEK293 cells. **a** Representative Western blots of surface-biotinylated proteins detected with a polyclonal anti-OATP1B1 antibody (75 kDa). HEK293 cells transfected with empty vector (Vector) were used as negative control. The same blot was also probed with an antibody against the plasma membrane marker Na^+/K^+-ATPase α-subunit (103 kDa) as a surface protein loading control. **b** Quantification of surface expression normalized to the loading control Na^+/K^+-ATPase. Relative intensities are presented as a percentage of wild-type OATP1B1 (OATP1B1). Band intensities at five or six different exposure times were quantified using ImageJ to ensure that the signal was within the linear range of the film. Each bar is mean ± SD of three independent experiments

estrone-3-sulfate and BSP (Fig. 3c). It is important to emphasize that all of these experiments were performed at a single time point and at a single substrate concentration within the initial linear range. Although such experiments can yield important information about changes in the transport activities of the tested proteins, kinetic analyses are needed to determine whether the reduced transport function is due to changes in the level of expression (Fig. 2), the apparent affinity and/or the translocation rate across the membrane. Therefore, we performed kinetics with all three substrates and all 16 mutants.

Kinetic Characterization of Mutant OATPs

Kinetic parameters for the three model substrates, estradiol-17β-glucuronide, estrone-3-sulfate and BSP, are summarized for the extracellular mutants in Table 2, for the transmembrane mutants in Table 3, and for the intracellular mutants in Table 4. In order to better visualize the effects, we calculated and compared the relative K_m and V_{max} values of all mutants in Fig. 4. We discovered that there were some mutants that displayed almost no effects on K_m (R181A, K90A, K90R, H92A, H92R and R93K) or V_{max} (R57A, R57K, K361R, R181A, R181H, K90A and R93A) values for all three substrates tested and some mutants that showed substrate-independent decreases in K_m (R580K and R580H) or V_{max} (R580K, R580H, H92A, H92K, H92R and R93K) values. However, seven of the 16 mutants showed substrate-dependent effects on the K_m values (R57A, R57K, K361A, R181H, R580A, H92K and R93A), and three of the mutants showed a substrate-dependent effect on the V_{max} values (K361A, R580A and K90R). Among these mutants that exhibited substrate-dependent effects, two (the extracellular K361A and the transmembrane R580A) affected both K_m and V_{max} in a substrate-dependent way.

The Extracellular Mutants of R57 and K361

Kinetics for R57 and K361 that are predicted at the extracellular side of OATP1B1 are shown in Table 2. The major effects of mutations at R57 compared to wild-type OATP1B1 were on the apparent affinities for the different substrates. Replacing the arginine by an alanine increased the K_m value for estradiol-17β-glucuronide by 5.7-fold and the value for BSP by 1.5-fold (Table 2). Replacing the

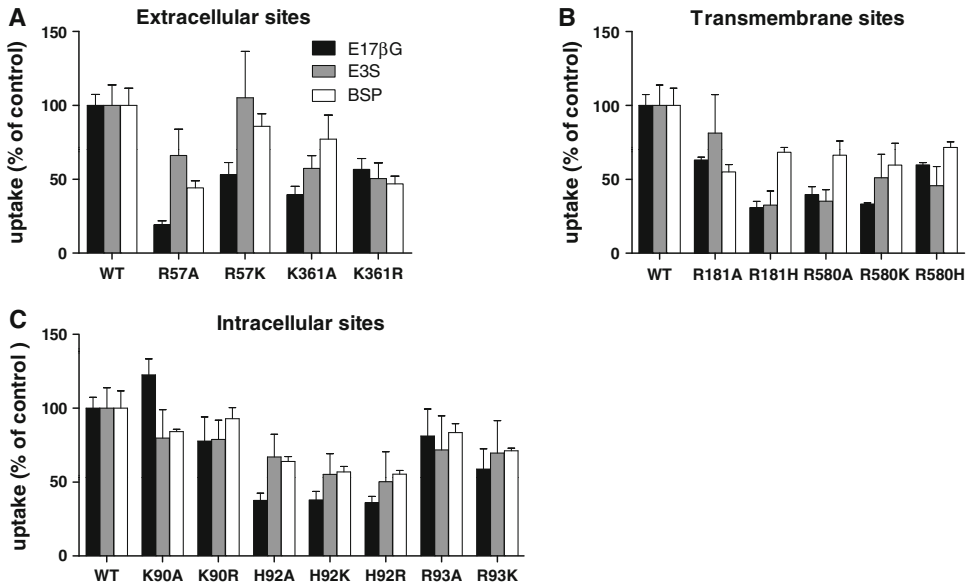

Fig. 3 Uptake rates of estradiol-17β-glucuronide (E17βG), estrone-3-sulfate (E3S) and bromosulfophthalein (BSP) mediated by wild-type OATP1B1 and its mutants. Uptake of 1 μM [^{3}H]-E17βG (*black bar*) and 0.05 μM [^{3}H]-BSP (*white bar*) was measured at 37°C for 1 min; uptake of 0.1 μM [^{3}H]-E3S (*gray bar*) was measured at 37°C for 30 s with HEK293 cells that were transfected with empty vector, OATP1B1 wild-type, or extracellular (**a**), transmembrane (**b**) and intracellular (**c**) mutants. Results were normalized for total protein expression in each well and presented as percentage of wild-type OATP1B1 (WT). Each bar is the mean ± standard error (SE) of five or six independent experiments with triplicate determinations in each experiment

Table 2 Kinetic parameters of wild-type OATP1B1 and extracellular mutants

Substrate	Mutant	K_m (μM)	V_{max} (pmol/[mg min])
E17βG	WT	5.35 ± 0.54	228 ± 19.8
	R57A	30.5 ± 3.64*	319 ± 50.6
	R57K	8.62 ± 0.33*	152 ± 31.3
	K361A	14.5 ± 2.02*	178 ± 36.0
	K361R	11.9 ± 2.57*	224 ± 24.3
E3S	WT	0.55 ± 0.12	38.6 ± 5.81
	R57A	0.81 ± 0.03	30.2 ± 5.53
	R57K	0.96 ± 0.21	22.4 ± 3.64
	K361A	0.54 ± 0.07	11.34 ± 2.95*
	K361R	1.83 ± 0.33*	23.9 ± 5.90
BSP	WT	0.46 ± 0.04	52.2 ± 9.42
	R57A	0.69 ± 0.04*	38.5 ± 4.69
	R57K	0.40 ± 0.04	38.3 ± 4.34
	K361A	0.97 ± 0.14*	52.4 ± 17.6
	K361R	1.05 ± 0.15*	51.7 ± 23.7

Kinetic parameters of wild-type and mutated OATP1B1-mediated E17βG (estradiol-17β-glucuronide), E3S (estrone-3-sulfate) and BSP (bromosulfophthalein) uptake were measured under initial linear rate conditions, corrected for surface expression and presented as mean ± SE of three to 13 independent experiments. Uptake of E17βG (1–50 μM) and BSP (0.05–3 μM) was measured at 37°C for 1 min; uptake of E3S (0.05–2 μM) was measured at 37°C for 30 s

* $P < 0.05$

Table 3 Kinetic parameters of wild-type OATP1B1 and transmembrane mutants

Substrate	Mutant	K_m (μM)	V_{max} (pmol/[mg min])
E17βG	WT	5.35 ± 0.54	228 ± 19.8
	R181A	5.48 ± 1.07	211 ± 6.71
	R181H	9.65 ± 1.95*	176 ± 32.5
	R580A	8.86 ± 1.04*	539 ± 93.2*
	R580K	0.52 ± 0.18*	17.8 ± 1.55*
	R580H	2.10 ± 0.12*	77.1 ± 3.88*
E3S	WT	0.55 ± 0.12	38.6 ± 5.81
	R181A	0.54 ± 0.09	37.0 ± 2.28
	R181H	0.24 ± 0.14	15.7 ± 9.27
	R580A	0.19 ± 0.01	29.0 ± 6.93
	R580K	0.10 ± 0.04*	7.27 ± 3.26*
	R580H	0.18 ± 0.04	11.8 ± 4.88*
BSP	WT	0.46 ± 0.04	52.2 ± 9.42
	R181A	0.49 ± 0.08	53.0 ± 8.01
	R181H	0.37 ± 0.12	48.1 ± 10.4
	R580A	0.42 ± 0.06	106 ± 20.7*
	R580K	0.21 ± 0.04*	12.5 ± 2.67*
	R580H	0.22 ± 0.05*	23.4 ± 4.78

Kinetics experiments were done under the same conditions as described in Table 2

Table 4 Kinetic parameters of wild-type OATP1B1 and intracellular mutants

Substrate	Mutant	K_m (μM)	V_{max} (pmol/[mg min])
E17βG	WT	5.35 ± 0.54	228 ± 19.8
	K90A	4.82 ± 0.96	162 ± 15.6
	K90R	4.72 ± 0.94	120 ± 12.4*
	H92A	3.63 ± 0.97	77.5 ± 11.2*
	H92K	5.41 ± 1.57	92.5 ± 4.12*
	H92R	6.53 ± 1.79	87.9 ± 20.8*
	R93A	4.76 ± 0.65	134 ± 35.3
	R93K	4.58 ± 1.23	85.5 ± 16.4*
E3S	WT	0.55 ± 0.12	38.6 ± 5.81
	K90A	0.43 ± 0.10	19.6 ± 5.09
	K90R	0.33 ± 0.08	22.3 ± 3.41
	H92A	0.17 ± 0.02	19.1 ± 4.77
	H92K	0.13 ± 0.03*	15.8 ± 6.37*
	H92R	0.16 ± 0.05	15.0 ± 6.86*
	R93A	0.21 ± 0.04	18.3 ± 7.00
	R93K	0.23 ± 0.06	17.5 ± 8.37
BSP	WT	0.46 ± 0.04	52.2 ± 9.42
	K90A	0.47 ± 0.15	35.6 ± 10.5
	K90R	0.63 ± 0.09	76.4 ± 11.9
	H92A	0.36 ± 0.16	25.5 ± 5.75
	H92K	0.22 ± 0.08*	16.5 ± 2.11
	H92R	0.55 ± 0.17	34.8 ± 3.48
	R93A	0.23 ± 0.05*	25.1 ± 2.05
	R93K	0.33 ± 0.14	23.6 ± 6.45

Kinetics experiments were done under the same conditions as described in Table 2

arginine by the positively charged lysine resulted in a K_m value that was now only 1.6-fold higher for estradiol-17β-glucuronide and not significantly different for BSP (Table 2). These results suggest that a positive charge at position 57 is favorable for normal estradiol-17β-glucuronide and BSP binding or transport and that changing this amino acid residue to an alanine reduces transport significantly, especially at low substrate concentrations.

The V_{max} values for R57A and R57K were similar to wild-type OATP1B1 for estradiol-17β-glucuronide, estrone-3-sulfate and BSP (Table 2). Although expression of R57A at the surface was reduced by 30% and expression of R57K was increased to 120% (Fig. 2), these different expression levels did not affect V_{max} values significantly for all three different substrates. Therefore, R57 may somehow be involved in substrate recognition or translocation, especially for estradiol-17β-glucuronide and BSP. Overall, a positive charge at position 57 seems to be preferable for normal OATP1B1 expression and function, and this conclusion is supported by the fact that in all 11 human OATPs there is a positive charge at the position corresponding to R57 of OATP1B1 (arginine: OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP3A1, OATP4A1 and OATP5A1; lysine: OATP2A1, OATP2B1, OATP4C1 and OATP6A1).

We also noted substrate-dependent differences for mutations at position K361. An alanine at position 361 increased the apparent K_m values for estradiol-17β-glucuronide and BSP but not for estrone-3-sulfate. However, the V_{max} value for estrone-3-sulfate was three times lower, explaining the reduced uptake seen in Fig. 3a. An arginine at position 361 increased the apparent K_m values for all three substrates to 2.2- to 3.3-fold but did not affect the V_{max} values. Contrary to R57, the charge conserved mutation K361R did not improve the substrate binding affinity compared to the K361A mutation (Table 2). Therefore, the conserved lysine, rather than simply the positive charge at this position, seems to be important for OATP1B1 function. This conclusion is further supported by the fact that at position 361 lysine is conserved in all human OATPs except for OATP6A1, for which so far no functional data are available.

The Transmembrane Mutants R181 and R580

Because the expression of R181K was very low, we could not characterize its transport function in detail. Kinetics for R181 and R580 are summarized in Table 3. Replacing arginine at position 181 with an alanine did not affect the apparent K_m or V_{max} values for all three substrates tested. For R181H we found a 1.8-fold increase in the K_m value for estradiol-17β-glucuronide but no significant changes for the other two substrates. Similarly, the maximal transport rates for the three substrates were not significantly different from wild-type OAT1B1 (Table 3). Thus, mutating R181 to a histidine decreased the affinity for estradiol-17β-glucuronide, presumably because the aromatic imidazole ring of histidine at this position is less favorable for the binding or translocation of estradiol-17β-glucuronide. This conclusion is supported by the observation that the arginine at position 181 is only conserved within the OATP1 family and a histidine is only found in members of the OATP4A1 family, transporters that are much less effective in mediating uptake of estradiol-17β-glucuronide (Tamai et al. 2000).

At position R580, a residue that has been shown to be important for BSP transport in OATP1B3 (Glaeser et al. 2010), K_m and V_{max} values for the three different substrates were affected in different ways. For the alanine replacement, the K_m value for estradiol-17β-glucuronide increased 1.7-fold, decreased for estrone-3-sulfate to 35% ($P = 0.069$) and did not change for BSP (Table 3). A similar pattern was seen for the effect on V_{max} values.

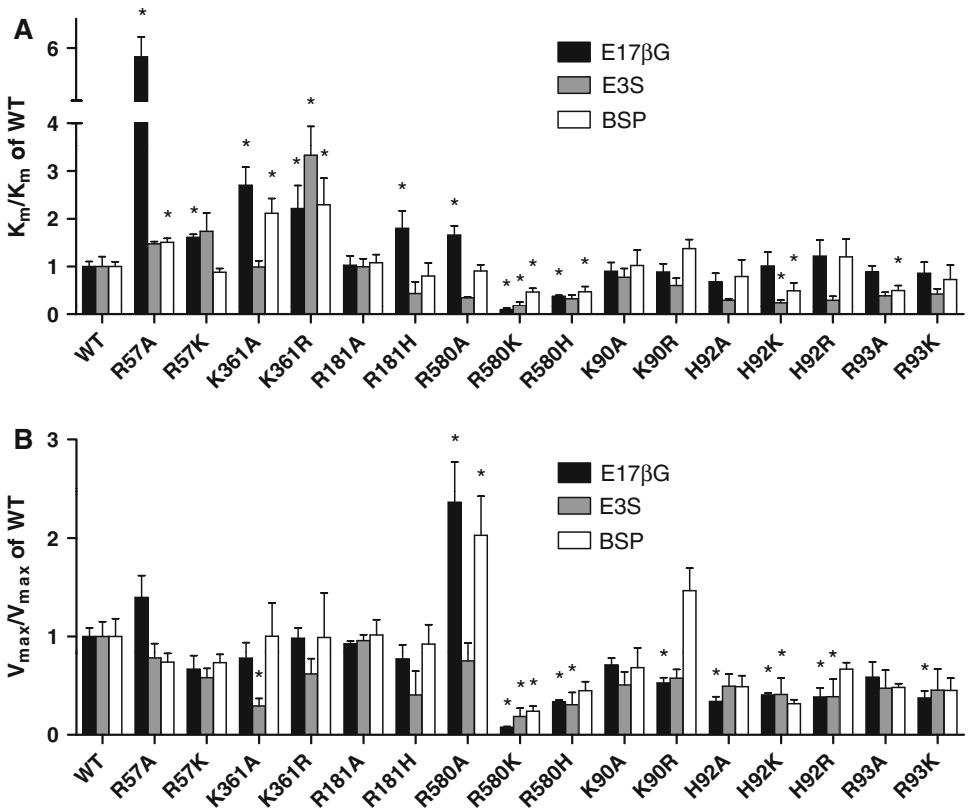

Fig. 4 Apparent K_m and V_{max} values relative to wild-type OATP1B1. K_m (**a**) and V_{max} (**b**) values summarized in Tables 2, 3, 4 were normalized to the values obtained with OATP1B1

Increases of 2.4- and 2.0-fold in the V_{max} values were seen for estradiol-17β-glucuronide and BSP, respectively. The V_{max} for estrone-3-sulfate was not changed. Replacing the arginine at position 580 by a lysine or histidine increased the apparent affinities (reduced K_m values) for all three substrates ($P = 0.065$ for estrone-3-sulfate), with a parallel decrease in the respective maximal transport rates (Table 3). Thus, the increased apparent affinities might have resulted in the decreased maximal transport rates. Such decreased maximal transport rates, corrected for surface expression, indicate that the turnover number or translocation rate is decreased, possibly due to a higher affinity of the substrate to the transporter and, therefore, less efficient release at the intracellular side. In order to investigate this further, we will have to isolate membrane vesicles and perform detailed kinetic experiments with exactly controlled substrate concentrations on both sides of the membrane. Such experiments are not possible with whole cells and were therefore not included in these studies. Therefore, R580 might be involved in either directly facilitating the substrate translocation or maintaining a correct conformation which is required for the translocation process. Previous studies with rat Oatp2a1 have shown that replacing R560, the arginine at the corresponding position of R580 in TM11, with the neutral asparagine abolished transport activity. However, substitution with the positively charged lysine resulted in a comparable K_m but a greatly decreased V_{max} value (Chan et al. 2002), indicating that arginine might be important in substrate translocation. Furthermore, the recent publication by Glaeser et al. (2010) has shown that R580K and R580G mutations in OATP1B3 also decreased the V_{max} values for BSP transport, while their K_m values were slightly increased. In addition to the consistent observations in several OATPs/Oatps, the importance of an arginine at position 580 is supported by the absolute conservation of this arginine residue at this position within all 11 human OATPs.

The Intracellular Mutants K90, H92 and R93

Mutations of the three intracellular residues K90, H92 and R93 had little substrate-dependent effect. Besides decreased K_m values for estrone-3-sulfate (H92K) and BSP (H92K and R93A), the major effects were seen on the V_{max} values for estradiol-17β-glucuronide and estrone-3-sulfate (Table 4). The maximal transport rates for estradiol-17β-glucuronide were reduced for all the intracellular mutants, although two were not statistically significant (K90A, $P = 0.14$, and R93A, $P = 0.053$). For estrone-3-sulfate transport R93K and H92R showed a significantly reduced V_{max} value but all other mutants had also reduced V_{max} values ($P = 0.068$–0.10), while for BSP uptake only the V_{max} for H92K was reduced ($P = 0.061$). Thus, these

intracellular positively charged amino acids seem to be more important for normal translocation of estradiol-17β-glucuronide and estrone-3-sulfate than for BSP. Being part of a short intracellular loop, they might interact as some kind of gate for the transport of certain substrates.

The major findings of this study are (1) that some of the positively charged amino acids within the predicted binding pocket indeed are important for normal OATP1B1-mediated transport of estradiol-17β-glucuronide, estrone-3-sulfate and BSP and (2) that several of the tested mutations affected OATP1B1 function in a substrate-dependent way. Such substrate-dependent effects have also been observed when naturally occurring polymorphisms were investigated. OATP1B1*1b (388A>G, N130D) and OATP1B1*5 (521T>C, V174A) are the two most frequently observed polymorphisms (König et al. 2006; Nozawa et al. 2002; Tirona et al. 2001). In vitro, N130D does not affect surface expression of the protein or transport of estradiol-17β-glucuronide and estrone-3-sulfate (Iwai et al. 2004; Kameyama et al. 2005; Tirona et al. 2001), but it reduces uptake of rifampicin and taurocholate (Michalski et al. 2002; Tirona et al. 2003) and increases uptake of BSP (Michalski et al. 2002). In vivo, N130D correlates with lower serum levels of ezetimibe, pravastatin and tacrolimus (Elens et al. 2007; Maeda et al. 2006; Oswald et al. 2008) and with an increased oral clearance of torasemide (Vormfelde et al. 2008). Thus, compared to wild-type OATP1B1, OATP1B1*1b has unchanged or increased or decreased transport rates depending on the substrate that is transported. The polymorphism V174A alone or together with N130D (OATP1B1*15) decreases clearance and increases plasma concentration of pravastain, simvastatin, ezetimibe, SN-38 and bilirubin (Han et al. 2008; Ieiri et al. 2004; Neuvonen et al., 2008; Niemi et al. 2004; Nishizato et al. 2003; Nozawa et al. 2005; Oswald et al. 2008; Pasanen et al. 2008; van der Deure et al. 2008; Zhang et al. 2007). In vitro studies using transiently transfected cell lines suggest that the decreased transport activity of OATP1B1*15 may be due to decreased protein expression on the cell surface (Kameyama et al. 2005) or due to decreased turnover number of the mutated transporter (Iwai et al. 2004).

In conclusion, for proteins with unknown structure and transport mechanisms, comparative modeling combined with the analysis of conserved amino acid residues is a powerful tool to identify candidate residues that may play important roles in structure and/or function of a transporter. Site-directed mutagenesis provided useful structural and mechanistic information for the bacterial transporters in the major facilitator superfamily, lactose permease (Abramson et al. 2003), glycerol-3-phosphate transporter (Huang et al. 2003) and multidrug transporter EmrD (Yin et al. 2006) prior to their crystallization. Because of the mainly anionic nature of OATP1B1 substrates, in this study we used site-directed mutagenesis to investigate the roles of several conserved positively charged amino acids facing the putative binding pocket of OATP1B1. We have demonstrated the important role of the positive charge at R580 for surface expression. In addition, substrate-dependent effects were seen for the extracellular mutants at R57 and K361 mainly on the K_m values, for the transmembrane R181 and R580 on both the K_m and V_{max} values and for the intracellular K90, H92 and R93 mainly on the V_{max} values, suggesting the possibility of multiple binding sites for the different substrates. These findings provided experimental validation for the prediction of the computer model and are important steps toward elucidating the structure–function relationship of OATP1B1.

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