Human organic anion transporter 1 (hOAT1) belongs to a superfamily of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-human immunodeficiency virus therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. Previously we suggested that the predicted transmembrane domain 1 (TM1) of hOAT1 might be important for its function. In the present study, we examined the role of each residue within TM1 of hOAT1 in substrate recognition and transport. Alanine scanning was used to construct mutants of hOAT1, and the uptake of model substrate para-aminomhippurate was studied in COS-7 cells expressing the mutant transporters. This approach led to the discovery of two critical amino acid residues, Leu-30 and Thr-36, in hOAT1. Amino acid substitution at Leu-30 or Thr-36 resulted in a complete loss of transport activities. We then further characterized Leu-30 and Thr-36 by mutagenizing these residues to amino acids with different physicochemical properties. Leu-30 was replaced with amino acids with varying sizes of side chains, including glycine, valine, and isoleucine. We showed that progressively smaller side chains at position 30 increasingly impaired hOAT1 function mainly because of the impaired surface expression of the transporter. Thr-36, another critical amino acid in TM1, was replaced by serine and cysteine. Similar to the substitution of Thr-36 by alanine, substitution by serine and cysteine at this position abolished transport activity without affecting the surface expression of the transporter. The fact that Thr-36 cannot be substituted with serine and that the side chains of alanine, serine, and cysteine are smaller than that of threonine by a methyl group indicate that both the methyl group and the hydroxyl group of Thr-36 could be critical for hOAT1 activity. Together we conclude that Leu-30 and Thr-36 play distinct roles in hOAT1 function. Leu-30 is important in targeting the transporter to the plasma membrane. In contrast, Thr-36 is critical for substrate recognition. The present study provided the first molecular evidence that transmembrane domain 1 is a critical determinant of hOAT1 function and may provide important insights into the structure-function relationships of the organic anion transporter family.

Organic anion transporters (OAT) play essential roles in the body disposition of clinically important anionic drugs including anti-human immunodeficiency virus therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (1). Several OAT isoforms have been cloned by us and others (2). OAT1 and OAT3 are predominantly expressed in the kidney and the brain. In the kidney, these transporters utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit/elimination across the apical membrane into the urine. Through this tertiary transport mechanism, Na\(^{+}\)K\(^{-}\)ATPase maintains an inwardly directed (blood-to-cell) Na\(^{+}\) gradient. The Na\(^{+}\) gradient then drives a sodium dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the Na\(^{+}\) gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell. OAT4 is present mainly in the placenta and the kidney. In the kidney, OAT4 functions as an organic anion/dicarboxylate exchanger at the apical membrane of the proximal tubule and is responsible for the reabsorption of organic anions driven by an outwardly directed dicarboxylate gradient (3). OAT2 is predominantly expressed in the liver (1). The transport mechanism for OAT2 remains to be elucidated.

Computer modeling based on hydropathy analysis predicted that these proteins have 12 putative membrane-spanning domains and multiple consensus sites for glycosylation and phosphorylation (1, 2). In a previous study (4) on the role of glycosylation in OAT1 function, our laboratory investigated the effect of disrupting the putative glycosylation sites in a mouse organic anion transporter (mOAT1) as well as its human counterpart hOAT1 by mutating asparagine (N) to glutamine (Q) and assessing the mutant transporters in HeLa cells. One of the key findings from that study is that the putative glycosylation site Asn-39 in mOAT1 was not glycosylated, but the corresponding site (Asn-39) in hOAT1 was glycosylated. Disrupting Asn-39 resulted in a complete loss of transport activity in both mOAT1 and hOAT1 without affecting their cell surface expression, suggesting that the loss of function is not because of deglycosylation of Asn-39 per se but rather is likely because of the change of this important amino acid critically involved in the substrate binding. Because Asn-39 in mOAT1 is located in the same position as Asn-39 in hOAT1, two amino acid residues from the mouth of the transmembrane domain 1 (TM1), we
therefore hypothesize that TM1 may be critical for hOAT1 function.

In the present study, we investigated the importance of TM1 in hOAT1. We identified two critical amino acid residues Leu-30 and Thr-36, which play distinct roles in hOAT1 function.

MATERIALS AND METHODS

Materials—[14C]-Aminoohippuric acid (PAH) was from PerkinElmer Life Sciences. Sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS-biotin) and streptavidin-agarose beads were purchased from Pierce. All other reagents were purchased from Sigma.

Site-directed Mutagenesis—Mutant transporters were generated by site-directed mutagenesis using hOAT1-myc as a template. hOAT1-myc contains a 10-amino acid c-myc tag at the C terminus of hOAT1. Previous studies from our laboratory (4) showed that the myc-tagged protein retained the functional properties of the native (unmodified) structure. The mutant sequences were confirmed by the dye-oligo chain termination method.

Expression in COS-7 Cells—COS-7 cells were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Confluent cells were transfected with DNA plasmid using LipofectAMINE 2000 reagent (Invitrogen) following the established protocol (5). Transfected cells were incubated for 14–20 h at 37 °C and then used for transport assay and cell surface biotinylation.

Transport Measurements—For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/CM (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4, 1.4 mM KH2PO4, 0.1 mM CaCl2, and 1 mM MgCl2, pH 7.3) and [14C]PAH. As shown in Table I, significant efflux of intracellular [14C]PAH was observed with both wild-type hOAT1 and its mutants when cells were exposed to medium containing an exchangeable substrate α-ketoglutarate, whereas little efflux was observed with the medium lacking α-ketoglutarate. These mutants were also as sensitive to the inhibition by probenecid as wild-type hOAT1 is; probenecid is an inhibitor for OAT1 (Table III). These results suggest that the substitution by alanine, substitution by serine or cysteine as wild-type hOAT1 is; probenecid is an inhibitor for OAT1 (Table III). These results suggest that the substitution by alanine, substitution by serine or cysteine.

**RESULTS**

Alanine Scanning of Residues in TM1—The proposed transmembrane domain 1 consists of 22 amino acid residues from Phe-16 to Leu-37. To probe the contribution of each residue to hOAT1 function, we systematically mutated each residue to alanine except for the pre-existing alanine at position 32, which was replaced by valine. The functional properties of these mutants were then determined by measuring the uptake of [14C]PAH in mutant-transfected COS-7 cells. A summary of data obtained from this analysis is shown in Table I. Although most of the mutants exhibited significant transport activities compared with the wild-type hOAT1, mutants L30A and T37A completely lost transport functions.

Functional Analysis of the Effect of Mutations in TM1—Wild-type OAT1 is known to function as an exchanger (1) with one organic anion being transported into the cells in exchange for another organic anion being effluxed out of the cells. To determine whether mutations of the residues in TM1 affect this functional characteristic, cells expressing hOAT1 mutants were preloaded with [14C]PAH followed by exposure to medium with or without α-ketoglutarate, another substrate for OAT1. As shown in Table II, significant efflux of intracellular [14C]PAH was observed with both wild-type hOAT1 and its mutants when cells were exposed to medium containing an exchangeable substrate α-ketoglutarate, whereas little efflux was observed with the medium lacking α-ketoglutarate. These mutants were also as sensitive to the inhibition by probenecid as wild-type hOAT1 is; probenecid is an inhibitor for OAT1 (Table III). These results suggest that the substitution by alanine at these positions preserves the basic functional characteristics of the wild-type hOAT1. Because L30A and T36A are nonfunctional, they were not included in these studies.

Effect of Mutations at Leu-30 and Thr-36 on hOAT1 Function—Because of the total loss in the uptake of PAH by L30A and T36A, additional studies were focused on these two residues. To elucidate the molecular mechanisms underlying the effects of mutations at Leu-30 and Thr-36, we mutagenized these two residues to amino acids with different physicochemical properties (Table IV). Leu-30 was replaced by amino acids with varying sizes of hydrophobic side chains, including glycine, valine, and isoleucine. As shown in Fig. 1a, substitution of Leu-30 with amino acids with large side chains such as valine and isoleucine resulted in detectable transport activities, although transport activities were reduced compared with that of wild-type hOAT1. In contrast, substitution of Leu-30 with amino acids with small side chains such as glycine and alanine rendered hOAT1 nonfunctional. This finding clearly demonstrates that progressively smaller side chains at position 30 increasingly impair hOAT1 function. Next, Thr-36 was replaced by the hydroxyl- or sulfur-containing polar residues serine or cysteine (Fig. 1b). Similar to the substitution by alanine, substitution by serine or cysteine

| Mutants | [14C]PAH uptake |
|---------|----------------|
| hOAT1-Wt | 100.0 ± 1.0 |
| L16A | 55.5 ± 1.4 |
| Q17A | 60.0 ± 7.4 |
| Q18A | 105.0 ± 20.0 |
| I19A | 95.7 ± 11.0 |
| Q20A | 50.0 ± 6.5 |
| V21A | 122.0 ± 11.0 |
| T22A | 120.4 ± 18.0 |
| L23A | 110.2 ± 16.2 |
| V24A | 102.0 ± 17.2 |
| V25A | 59.0 ± 6.8 |
| L26A | 70.0 ± 9.9 |
| P27A | 105.3 ± 5.3 |
| L28A | 53.7 ± 5.4 |
| L29A | 55.5 ± 8.2 |
| L30A | 5.0 |
| M31A | 120.0 ± 13.1 |
| A32V | 62.9 ± 11 |
| S33A | 103.0 ± 18.0 |
| H34A | 50.4 ± 6.2 |
| N35A | 50.0 ± 4.4 |
| T36A | 0.0 |
| L37A | 54.0 ± 7.0 |

Critical Amino Acids in Transmembrane Domain 1 of hOAT1
that measured in the control group. The results shown are means ± S.E. (n = 3).

### TABLE II
**Efflux of [14C]PAH**

| Mutants       | Intracellular [14C]PAH remaining % of control |
|---------------|-----------------------------------------------|
| hOAT1-Wt      | 350 ± 70                                      |
| F16A          | 590 ± 10.0                                    |
| Q17A          | 360 ± 70                                      |
| Q18A          | 330 ± 7.0                                     |
| I19A          | 320 ± 7.0                                     |
| Q20A          | 310 ± 7.0                                     |
| V21A          | 300 ± 7.0                                     |
| T22A          | 290 ± 7.0                                     |
| L23A          | 280 ± 7.0                                     |
| V24A          | 270 ± 7.0                                     |
| L26A          | 260 ± 7.0                                     |
| P27A          | 250 ± 7.0                                     |
| L28A          | 240 ± 7.0                                     |
| M31A          | 230 ± 7.0                                     |
| A32V          | 220 ± 7.0                                     |
| S33A          | 210 ± 7.0                                     |
| H34A          | 200 ± 7.0                                     |
| N35A          | 190 ± 7.0                                     |
| L37A          | 180 ± 7.0                                     |

### TABLE III
**Inhibition of PAH uptake by probenecid**

Transport of PAH in COS-7 cells, expressing hOAT1 wild-type (Wt) and its mutants, was measured in the presence (experimental group) and absence (control group) of 0.5 mM probenecid. Uptake activity remaining in the experimental group was expressed as a percentage of that measured in the control group. The results shown are means ± S.E. (n = 3).

| Mutants       | Uptake remaining % of control |
|---------------|-------------------------------|
| hOAT1-Wt      | 350 ± 7.0                     |
| I19A          | 320 ± 7.0                     |
| Q20A          | 290 ± 7.0                     |
| V21A          | 270 ± 7.0                     |
| T22A          | 250 ± 7.0                     |
| L23A          | 240 ± 7.0                     |
| V24A          | 230 ± 7.0                     |
| L26A          | 220 ± 7.0                     |
| P27A          | 210 ± 7.0                     |
| L28A          | 200 ± 7.0                     |
| M31A          | 190 ± 7.0                     |
| A32V          | 180 ± 7.0                     |
| S33A          | 170 ± 7.0                     |
| H34A          | 160 ± 7.0                     |
| N35A          | 150 ± 7.0                     |
| L37A          | 140 ± 7.0                     |

at this position resulted in a nonfunctional transporter.

**Immunoblot Analysis of the Effect of Mutations at Leu-30 and Thr-36 on Cell Surface Expression of hOAT1**—The reduced or lack of transport activities of mutant transporters could be caused by changes in the absolute number of transporters, turnover rate, substrate binding affinity, or a combination of these factors. As a first step in evaluating the possible changes, we compared the protein expression levels of wild-type hOAT1 and its mutants on the cell surface (Fig. 2) and in the total cell extracts (data not shown) by immunoblot analysis. Although the amount of total cellular proteins of the Leu-30 mutants (L30G, L30A, L30V, and L30I) were similar to that of the wild type, the membrane abundance of these mutants decreased significantly in mutant-transfected cells as compared with that of wild-type hOAT1-transfected cells; the smaller the side chains at position 30, the less expression of the mutants at the cell surface (Fig. 2a). In sharp contrast with the mutants of Leu-30, substitution of Thr-36 with alanine, serine, and cysteine had no effect on its surface expression (Fig. 2b). All of the mutants of Thr-36 had a similar amount of surface expression as compared with the wild type.

**Immunofluorescence Analysis of the Effect of Mutations at Leu-30 and Thr-36 on Cell Surface Expression of hOAT1**—The effect of mutations at Leu-30 and Thr-36 on the cellular distribution of hOAT1 was further investigated by immunofluorescence analysis (Fig. 3). Although the plasma membrane was clearly labeled (shown as green fluorescence) in cells transfected with wild-type hOAT1 as well as with all of the Thr-36 mutants (Fig. 3b), fluorescence remained mainly in the intracellular compartment in cells transfected with most of the Leu-30 mutants (L30G, L30A, and L30V) (Fig. 3a). Phase contrast images showed that cells were fully attached to the culture dishes under all conditions. Considering these data together, it can be concluded that the proper targeting of hOAT1 to the plasma membrane was impaired by the substitution at position 30 but not by the substitution at position 36.

**Kinetic Analysis of PAH Transport Mediated by Mutant L30V**—To further examine the mechanisms underlying the reduced transport activity of hOAT1 by substitution of Leu-30 with valine, we determined [14C]PAH uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 4) showed that a valine substitution of Leu-30 resulted in decreased Vₘₐₓ (110.8 ± 3.1 pmol/μg 3 min with wild-type hOAT1, 25.5 ± 0.5 pmol/μg 3 min with L30V) and decreased Kₘ (14.8 ± 0.1 μM with wild-type hOAT1, 7.8 ± 0.4 μM with L30V).

**DISCUSSION**

The vital importance of organic anion transport in body drug disposition is clear (1, 2). However, our knowledge about the structure-function relationships of organic anion transporters is minimal. Our laboratory has recently carried out a study on the role of glycosylation in OAT1 function (4). In that study, we
To further investigate the significance of Leu-30 and Thr-36, we then mutagenized these two residues to several other amino acids with different physicochemical properties (Table 4). Leu-30 was replaced by amino acids with varying sizes of hydrophobic side chains such as glycine, alanine, valine, and isoleucine. We showed that the side chain size of the residues at position 30 is of functional significance provided that no charge is present. When native Leu 30 was replaced with valine and isoleucine, residues with side chains slightly smaller than or similar to that of leucine and equally neutral, the resulting hOAT1 mutants (L30V and L30I) still exhibited measurable transport activities, although transport activities were reduced compared with that of wild-type hOAT1. Isoleucine is a stereoisomer of leucine. Therefore, the steric difference between isoleucine and leucine may also contribute to the functional difference between L30I and the wild-type hOAT1. In contrast, when native Leu-30 was replaced with glycine or alanine, residues with much smaller side chains than leucine and devoid of charge, the resulting hOAT1 mutants (L30G, and L30A) became nonfunctional.

Thr-36, another critical amino acid identified in TM1, was replaced by serine or cysteine. Similar to the substitution by alanine, substitution by serine or cysteine resulted in a nonfunctional transporter. The hydroxyl group on threonine and serine often participates in substrate binding or maintaining the protein structure through forming hydrogen bonds (6–8). If this is true, then one might expect that the substitution of Thr-36 by serine would rescue the loss of hOAT1 function that resulted from the substitution by alanine. However, this recovery of the function by serine substitution was not observed in our study. On the other hand, the size of the side chains of alanine, serine, and cysteine are smaller than that of threonine by one methyl group. Because alanine itself could not rescue the function when substituted at this position, a methyl group alone is apparently not sufficient to recapitulate the activity. Therefore, it is possible that both the methyl group and the hydroxyl group presented on threonine play critical roles in maintaining hOAT1 function.

The reduced or lack of transport activity in mutants of Leu-30 and Thr-36 could result from the reduced or lack of expression of the mutant transporter protein or could result from impaired binding abilities of the mutants for their substrates. By directly measuring cell surface expression of these mutants (Figs. 2 and 3), we observed two distinct phenomena. By directly measuring cell surface expression of these mutants (Figs. 2 and 3), we observed two distinct phenomena.
resulted in a dramatic decrease in the expression of the mutants at the cell surface (despite the similar total cell expression of these mutants relative to that of wild-type hOAT1, data not shown). In sharp contrast, the replacement of Thr-36 by alanine, serine, and cysteine had no effect on the surface expression of the transporter. These results suggest that the trafficking of the transporter to the plasma membrane was impaired by the substitution at position 30 but not by the substitution at position 36.

Finally, our kinetic analysis of L30V showed that the moderate transport activity of this mutant was contributed by a reduced surface expression (\( V_{\text{max}} \)) and an increased binding affinity (\( 1/K_m \)) as compared with that of the wild type.

In conclusion, Leu-30 and Thr-36 play distinct roles in hOAT1 function. Leu-30 is important in targeting the transporter to the plasma membrane. In contrast, Thr-36 is critical for substrate recognition. This is the first identification and characterization of critical amino acid residues in the membrane-spanning region of hOAT1 and may provide important insights into the structure-function relationships of the organic ion transporter family.

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