Topological assessment of oatp1a1: a 12-transmembrane domain integral membrane protein with three N-linked carbohydrate chains

Pijun Wang, Soichiro Hata, Yansen Xiao, John W. Murray, and Allan W. Wolkoff

Department of Medicine, Division of Hepatology, Marion Bessin Liver Research Center; and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York

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Wang P, Hata S, Xiao Y, Murray JW, Wolkoff AW. Topological assessment of oatp1a1: a 12-transmembrane domain integral membrane protein with three N-linked carbohydrate chains. Am J Physiol Gastrointest Liver Physiol 294: G1052–G1059, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00584.2007.—Organic anion transport protein 1a1 (oatp1a1), a prototypical member of the oatp family of highly homologous transport proteins, is expressed on the basolateral (sinusoidal) surface of rat hepatocytes. The organization of oatp1a1 within the plasma membrane has not been well defined, and computer-based models have predicted possible 12- as well as 10-transmembrane domain structures. Which of oatp1a1’s four potential N-linked glycosylation sites are actually glycosylated and their influence on transport function have not been investigated in a mammalian system. In the present study, topology of oatp1a1 in the rat hepatocyte plasma membrane was examined by immunofluorescence analysis using an epitope-specific antibody designed to differentiate a 10- from a 12-transmembrane domain model. To map glycosylation sites, the asparagines at the each of the four N-linked glycosylation consensus sites were mutagenized to glutamines. Mutagenized oatp1a1 constructs were expressed in HeLa cells, and effects on protein expression and transport activity were assessed. These studies revealed that oatp1a1 is a 12-transmembrane-domain protein in which the second and fifth extracellular loops are glycosylated at asparagines 124, 135, and 492, whereas the potential glycosylation site at asparagine 62 is not utilized, consistent with its position in a transmembrane domain. Constructs in which more than one glycosylation site were eliminated had reduced transport activity but not necessarily reduced transporter expression. This was in accord with the finding that fully unglycosylated oatp1a1 was well expressed but located intracellularly with limited transport ability as a consequence of its reduced cell surface expression.

RAT OATP1A1 IS A PROTEIN THAT is expressed on the basolateral plasma membrane of hepatocytes, where it mediates uptake of various organic anionic compounds in exchange for a counteranion such as bicarbonate (23) or GSH (14). It is the first described member of the family of organic anion transport proteins (oatps), a group of highly homologous hydrophobic proteins that transport a wide variety of amphipathic compounds (7, 16). Transport activity of oatp1a1 has been shown to be regulated posttranslationally by serine phosphorylation (4), and recent studies identified the sites of phosphorylation as being on two adjacent serine residues near the carboxy terminus (29). The organization of oatp1a1 within the plasma membrane has not been well defined. Although the oatps have been assumed to have 12-transmembrane domains, this has not been tested directly, and computer-based models have shown possible 12- as well as 10-transmembrane-domain structures. Although evidence has been presented that oatp1a1 is N-glycosylated (1, 13) and has four potential N-linked glycosylation sites (1, 10), which of these sites are utilized and their influence on transport function have not been investigated in a mammalian system, although a previous study in which this protein was expressed in Xenopus laevis oocytes was interpreted as showing that all four sites were glycosylated (13). If true, this would have major implications regarding oatp1a1 membrane topology, since it would imply an extracellular localization for the N-linked glycosylation consensus site at asparagine 62 that all other models imply is in a transmembrane domain and consequently unavailable to undergo glycosylation. In the present study, topology of oatp1a1 in the rat hepatocyte plasma membrane was assessed by immunofluorescence analysis using an epitope-specific antibody designed to differentiate a 10- from a 12-transmembrane-domain model. Glycosylation sites were mapped by using a mutagenesis protocol in which mutagenized oatp1a1 constructs were expressed in HeLa cells and effects on protein expression and transport activity were assessed. These studies revealed that oatp1a1 is a 12-transmembrane-domain protein in which the potential N-linked glycosylation sites at asparagines 124, 135, and 492 but not 62 are utilized.

MATERIALS AND METHODS

Hydropathy analysis. Prediction of transmembrane segments of oatp1a1 (accession number NP 058807) was performed by using TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), a program made available by The Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/index.shtml) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), a program made available by EMBnet (http://www.embnet.org/).

Mutagenesis of oatp1a1. N-linked glycosylation occurs at asparagines residues that lie in the following sequence: Asn-X-Thr/Ser where X is any amino acid but proline or aspartic acid (5). Based on this, oatp1a1 has four potential N-glycosylation sites at asparagine residues 62, 124, 135, and 492. Starting with a template consisting of oatp1a1 in the pSPORT1 vector as described previously (9), each of these asparagines (N) was mutated to a glutamine (Q), thus preventing its glycosylation, using the QuickChange site-directed mutagenesis kit (Stratagene). A plasmid containing each of the four potential N-linked glycosylation sites was generated for each of the four potential N-linked glycosylation sites, the respective asparagine (N) residue was mutated to glutamine (Q) with QuikChange site-directed mutagenesis kit (Stratagene), and the resulting construct was introduced into the pSPORT1 vector. The resulting plasmid containing each of the four potential N-linked glycosylation sites was generated for each of the four potential N-linked glycosylation sites, the respective asparagine (N) residue was mutated to glutamine (Q) with QuikChange site-directed mutagenesis kit (Stratagene), and the resulting construct was introduced into the pSPORT1 vector.
mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s directions. PCR primers that were used for mutagenesis are shown in Table 1. In some studies (Table 1), novel N-glycosylation consensus sites were introduced into oatp1a1, by using the identical strategy. For multiple mutations, changes were introduced successively from the NH₂ terminus to the COOH terminus. Plasmid DNA was extracted with the Qiagen Plasmid mini kit (Qiagen, Santa Clarita, CA), and mutations were verified by automated sequencing in the DNA Sequencing Facility of the Albert Einstein College of Medicine.

**Table 1. Site Directed Mutagenesis of oatp1a1**

<table>
<thead>
<tr>
<th>AA Mutation</th>
<th>Change in Nucleotide</th>
<th>Sense Primer</th>
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<tbody>
<tr>
<td>N62Q</td>
<td>AAT→CAA</td>
<td>5'GCTGGATTTAACAAGGAGCTTTGAG3'</td>
</tr>
<tr>
<td>N124Q</td>
<td>AAC→CAG</td>
<td>5'ACACACAGGGGACACTTGCCATCAA3'</td>
</tr>
<tr>
<td>N135Q</td>
<td>AAG→CAA</td>
<td>5'CTTGTATGAGAGCAAGACAGAAG3'</td>
</tr>
<tr>
<td>N492Q</td>
<td>AAT→CAA</td>
<td>5'GAGTCTATTGAGACACATGTCGAG3'</td>
</tr>
</tbody>
</table>

**Disrupted N-glycosylation consensus sites**

**Added N-glycosylation consensus sites**

| F597S       | TTC→AGC              | 5'ATAAACAGCTAGAGAGCATT3' |
| D231N       | GAC→AAC              | 5'GTTGAGATGACTGCTAGCT3' |
| L258S       | CTTG→AGT             | 5'GTTGATGACTGCTAGCT3' |
| K647N       | AAG→AAC              | 5'GCTGGAGAGGAGAAGGAGC3' |
| I519N       | ATT→AAT              | 5'TTAACACATCAGACTGACT3' |

Underlined nucleotides in the sense primer sequences are those that result in disrupted N-glycosylation consensus sites.

646–658 (EKESHTDVGHSP) near the COOH terminus of oatp1a1 (1). A cysteine residue was added to the carboxy terminus of each of these peptides during synthesis (Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine) to facilitate attachment to maleimide-activated KLH (Pierce) according to the manufacturer’s instructions. Specificity of antibodies was evaluated by immunoblot.

**Immunoblot analysis of oatp1a1 expression.** HeLa cells or rat liver homogenate were extracted with 0.1 M Na₂CO₃ to enrich the yield of integral membrane proteins (1). Proteins were resolved on 10% SDS-PAGE and immunoblots were performed by a chemiluminescence procedure using a rabbit antibody to oatp1a1 as we have described previously (1, 8). In some studies, proteins were N-deglycosylated by using N-glycosidase F (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions as we have described previously (1, 8).

**Immunofluorescence analysis.** Hepatocytes, isolated from rats as described previously (18, 19), were cultured overnight on coverslip-bottomed dishes (MatTek) coated with 0.5 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ) in Hepatocyte medium (In Vitro) and saturated with CO₂ (Invitrogen). A freeze-thawing procedure was used for permeabilization (15), because it was found that treatment with either Triton X-100 or digitonin detergents (0.1%) decreased the amount of oatp1a1 that was detectable by immunofluorescence. In this procedure, cells were frozen by exposure to −80°C for 5 min, then rapidly thawed, and immediately fixed by treatment with 4% formaldehyde in PBS for 20 min. Fixed cells were washed three times in PBS containing 5 mg/ml casein and then incubated in this solution for 10 min to block nonspecific binding. Cells were then treated for 50 min with primary antibody followed by washing and treatment with Cy2-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). They were then washed and incubated in anti-bleaching solution (PBS containing 50% glycerol and 2 mg/ml ascorbic acid). Nonpermeabilized hepatocytes were prepared by cooling cells to 4°C for 30 min followed by treatment with blocking buffer and primary and secondary antibody at 4°C. Wide-field microscopy images were captured on a ×60 Olympus objective, NA 1.4, mounted on an Olympus IX71 Microscope, with a CoolSnap HQ cooled charge-coupled device camera (Roper Scientific, Trenton, NJ) and Cy2 excitation and emission filters using Metamorph software (Molecular Devices, Sunnyvale, CA). Identical exposure times (200 ms) and brightness adjustments (image normalization) were used for the capture of images from permeabilized and nonpermeabilized cells.

In other studies, 293T cells that had been transfected with pcDNA3.1 plasmid alone or pcDNA3.1-oatp1a1 wild-type or mutant (N62,124,135,492Q) plasmids were cultured in DMEM containing 10% fetal bovine serum in coverslip-bottomed dishes precoated with 0.5 mg/ml Matrigel. After 2 days, cells were fixed in MEPS buffer (35 mM MgSO₄, 27.8 mM glucose, 2.5 mM CaCl₂, and 25 mM HEPES, pH 7.2). In brief, cells were washed three times with SFM and
incubated at 37°C or 4°C for 1 min in 1 ml of SFM containing 1 μM [35S]BSP. Cells were then washed five times with SFM at 4°C. The third wash contained 5% BSA and was allowed to stand for 5 min at 4°C. Cells were harvested and radioactivity was determined. Cell protein was determined in replicate plates by the BCA assay (Pierce) according to the manufacturer’s instructions with BSA as the standard. In saturation studies, varied concentrations of [35S]BSP (0.25–15 μM) were used. Experimental data was subjected to non-
linear least squares fit (SigmaPlot v. 6.1, SPSS, Chicago, IL) to the equation

\[ V = \frac{V_{\text{max}} \times [S]}{K_m + [S]} + k \times [S] \]

where \( V \) represents initial rate of uptake, \([S]\) is the concentration of ligand, \( K_m \) and \( V_{\text{max}} \) are the Michaelis-Menten constant and the maximum velocity, respectively, and \( k \) is a linear term representing diffusional (noncarrier mediated) uptake, as described previously (4, 8).

**RESULTS**

**Predicted topology of oatp1a1.** Two possible configurations of oatp1a1 were predicted by the computer modeling programs that were used. The TMHMM algorithm predicted 10-transmembrane domains (Fig. 1A), whereas the TMpred algorithm predicted 12 (Fig. 1B). To differentiate these possibilities, an antibody was prepared to the oatp1a1 sequence corresponding to amino acids 184–200. This sequence is present on the oatp1a1 extracellular domain in the 10-transmembrane domain model (Fig. 1A) and is on the cytosolic domain in the 12-transmembrane domain model (Fig. 1B). This antibody was used for immunofluorescence examination of rat hepatocytes with and without cell permeabilization. As seen in Fig. 2, staining for oatp1a1 was seen only when cells were permeabilized. In the absence of permeabilization, oatp1a1 was not detectable, consistent with intracellular localization of the antigenic epitope.

**N-glycosylation sites on oatp1a1.** Of the four N-glycosylation consensus sites on oatp1a1, three are in predicted extracellular domains [amino acids (aa) 124, 135, and 492] and one is in a predicted transmembrane segment at aa 62 (Fig. 1). Previous studies showed that in rat liver oatp1a1 is N-glycosylated (1). As seen in Fig. 3, it is also N-glycosylated in HeLa cells, with faster mobility on SDS-PAGE following incubation in N-glycosidase F, an enzyme that removes N-linked carbohydrate chains. Although these studies show that oatp1a1 is N-glycosylated, they do not provide information regarding which of the four potential sites are utilized. To examine this issue, a series of mutagenized constructs of oatp1a1 was prepared. Constructs were expressed in HeLa cells and migration of oatp1a1 on SDS-PAGE was used to evaluate changes in its glycosylation state. Initial studies were performed in preparations in which the specific glycosylation site was disrupted by mutation of the asparagine in the consensus sequence to a glutamine (N to Q). As seen in Fig. 4A, there was no effect on migration on SDS-PAGE of expressed oatp1a1 in which the first potential N-glycosylation site at N62 had been mutated to Q. Mutation of each of the three other potential N-glycosylation sites (asparagines 124, 135, and 492) resulted in faster migration of the expressed protein (Fig. 4A), consistent with loss of an N-linked carbohydrate chain from the protein. Effects of multiple mutations were also examined. Migration of expressed protein in which asparagines 62 and 124 were mutated to glutamine was identical to that of the protein with the single mutation at asparagine 124, consistent with the finding that oatp1a1 was N-glycosylated at amino acid 124, but not at amino acid 62 (Fig. 4A). The triple mutation in which asparagines 62, 124, and 135 were mutated to glutamines migrated faster, consistent with loss of two N-glycosylation groups, and the quadruple mutation in which all four potential N-glycosylation sites were mutated migrated still faster, consistent with loss of three N-glycosylation groups (Fig. 4C).

In additional studies, further mutations were performed to add new potential N-glycosylation sites to the backbone oatp1a1 construct lacking all of the four original potential N-glycosylation sites. Of these five mutagenized constructs, there was good expression of D231N, F597S, and K647N. Expression of L258S was diminished and there was no detectable expression of I519N (Fig. 4C). There was no evidence that any of these sites was glycosylated in the four forms that were expressed, since migration on SDS-PAGE was unaltered (Fig. 4C).
Effect of N-glycosylation site mutation on oatp1a1 transport function.

There was no effect on oatp1a1-mediated transport activity toward \[^{35}\text{S}]\text{BSP}\) of mutation of the N-linked glycosylation sites at asparagines (N) 124 or 135 (\(P = 0.4\), Fig. 4B), which lie on the second extracellular loop (Fig. 1B). Mutation of asparagine 492, eliminating the N-linked glycosylation site in the fifth extracellular loop, resulted in a 30% reduction in \[^{35}\text{S}]\text{BSP}\) uptake \((P = 0.04)\). However, an \(\approx 50\%\) reduction in \[^{35}\text{S}]\text{BSP}\) uptake \((P < 0.001)\) was seen with mutation of asparagine 62, in which a carbohydrate chain is not eliminated.

There was little effect of these single-amino acid mutations on oatp1a1 expression (Fig. 4A). Uptake of \[^{35}\text{S}]\text{BSP}\) was substantially reduced by 50% or more with multiple simultaneous mutations at these sites \((P < 0.001, \text{Fig. 4B, D})\). To see whether any of these mutations might have resulted in altered affinity of the transporter for \[^{35}\text{S}]\text{BSP}\), we did a survey of saturation kinetics as shown in Fig. 4D. Interestingly, there was little difference in \(K_m\), which varied between 1 and 2 \(\mu\text{M}\) for all constructs. With the exception of the N124Q and N135Q mutants, there were variable reductions in \(V_{\text{max}}\) for cells expressing the other mutants compared with cells expressing wild-type oatp1a1 (Fig. 4D), consistent with the possibility of reduced effective expression of these mutated forms of oatp1a1 at the cell surface. Additional studies were performed in which N-linked glycosylation consensus sites were mutated into the N62,124,135,492Q oatp1a1 construct, in which the naturally occurring consensus sites had been eliminated. As seen in Fig. 4B, none of these expressed mutated oatp1a1 constructs could mediate BSP uptake.

Effect of N-glycosylation site mutation on oatp1a1 subcellular distribution.

A previous study suggested that glycosylation of oatp1a1 is required for its transport activity \((13)\). Although this is possible, the studies presented above in which \(V_{\text{max}}\) was reduced are consistent with the possibility that changes in \[^{35}\text{S}]\text{BSP}\) uptake could be due to alterations in subcellular trafficking and consequent reduced transporter expression at the cell surface. This was examined by immunofluorescence microscopy, comparing cellular expression of wild-type oatp1a1 and oatp1a1 in which the four potential N-glycosylation sites had been mutated (N62,124,135,492Q). As seen in Fig. 5, wild-type transporter was abundant at the plasma membrane, whereas mutated transporter was localized...
Elucidation of the topology of oatp1a1 may have important potential implications for understanding regulation of its transport activity. Although it has been assumed on the basis of computer modeling that the members of the oatp family of proteins have 12-transmembrane domains (7), this has not been tested directly. As described above, a 10-transmembrane domain model, in which the fourth and fifth transmembrane segments of the 12-transmembrane domain model are extracellular, has also been predicted (Fig. 1A). In this model, amino acids 184–200 reside on a large extracellular loop as depicted in Fig. 1A. This sequence is intracellular in the 12-transmembrane domain model (Fig. 1B). To validate one of these models, a peptide-specific antibody was generated against this sequence and was used to examine interaction with oatp1a1 in hepatocytes by immunofluorescence with or without permeabilization of the plasma membrane. As seen in Fig. 2, this antibody recognized oatp1a1 only in hepatocytes in which the plasma membrane had been permeabilized. Permeabilization permits entry of antibody into the cell interior and implies that this segment of the protein is intracellular. Thus the predicted 10-transmembrane domain model cannot be correct. To further test the 12-transmembrane model, the location of N-linked glycosylation sites was examined.

As seen in Fig. 1, oatp1a1 has four potential N-linked glycosylation sites at asparagine residues 62, 124, 135, and 492. The fact that treatment of cell extracts with N-glycanase results in reduction in size on immunoblot (Fig. 3) implies that one or more of these potential glycosylation sites is utilized. Definition of glycosylation site usage provides important topological information, since glycosylation will occur only on extracellular domains of the protein (22). Mutation of each of these asparagines to a glutamine was performed, effectively disrupting the consensus sequence for N-glycosylation. Plasmids were transfected into HeLa cells, and expression of oatp1a1 was examined by immunoblot. As described above, these studies showed that there was glycosylation of oatp1a1 at asparagines 124, 135, and 492, but not at asparagine 62 (Fig. 4A). These results are in contrast with those of Lee et al. (13), who suggested that all four sites on oatp1a1 were glycosylated, implying that they were extracellular. This result would not fit any of the proposed models (Fig. 1), in which asparagine 62 lies within a transmembrane segment. There are several reasons for the discordant results. The previous study was performed by expressing wild-type and mutant constructs in Xenopus laevis oocytes and examining migration of oatp1a1 by immunoblot. It is possible that protein folding and glycosylation of oatp1a1 in this nonmammalian cell differ from that in mammalian cells. Such species differences are exemplified by the finding in that study that oatp1a1 expressed in transfected yeast was entirely nonglycosylated (13). It should also be noted that oatp1a1 mutations in the Lee et al. study consisted of substitution of asparagine by aspartic acid, a charged amino acid that could by itself lead to differences in migration on SDS-PAGE, making interpretation of the mutagenesis studies difficult.

As seen in Fig. 4B, uptake of [35S]BSP was substantially reduced in constructs incorporating multiple mutations of N-linked glycosylation sites. Previous studies of other plasma membrane glycoproteins indicated that N-glycosylation can be important for their proper folding and intracellular trafficking (20–22, 25, 30). For this reason, we examined the subcellular...
distribution in transfected HeLa cells of wild-type oatp1a1 and oatp1a1 in which all four potential N-glycosylation sites were mutated (Fig. 5). Total expression of this mutated protein was similar to that of the wild-type protein (Fig. 4C). However, in contrast to immunofluorescence results for HeLa cells expressing wild-type oatp1a1, there was little expression of mutated protein on the cell surface; rather, it was present in intracellular structures (Fig. 5). This failure to reach the cell surface was in accord with the finding that cells transfected with this mutated oatp1a1 had little or no uptake of $^{[35]}$BSBP compared with cells transfected with vector alone (Fig. 4B).

Lee et al. (13) expressed the corresponding oatp1a1 in which the four sites were mutagenized from asparagine to aspartic acid as a FLAG construct in Xenopus laevis oocytes. They also found reduced expression at the oocyte surface but provided no indication that the protein was actually expressed or accumulated intracellularly.

Results in the present study indicate that the second and fifth extracellular loops of oatp1a1 are glycosylated, whereas the potential glycosylation site at asparagine 62 is not utilized, consistent with its position in a transmembrane domain. Additional experiments were performed in which oatp1a1 was mutated, resulting in insertion of novel N-linked glycosylation consensus sites (Fig. 4, B and C). If such a site becomes glycosylated, this implies that it is extracellular. However, failure to become glycosylated does not provide meaningful information as to topological orientation. We started with the mutated oatp1a1 construct lacking all N-linked glycosylation consensus sites and added individual sites at residues 231 (extracellular), 258 (transmembrane), 519 (transmembrane), 597 (extracellular), and 647 (intracellular). Following expression of each of these constructs in HeLa cells there was no evidence on immunoblot for glycosylation (Fig. 4C). Expression of the constructs with mutations in the transmembrane domains was poor, and there was no detectable expression of the I519N mutant in repeated assays. None of these constructs was able to mediate $^{[35]}$BSBP uptake (Fig. 4B). It is possible that addition of a single glycosylation site to the oatp1a1 unglycosylated backbone is not sufficient to permit appropriate subcellular processing. Although further studies would be needed to clarify this issue, we can conclude on the basis of our studies that oatp1a1 has three N-linked carbohydrate chains and conforms to the 12- rather than 10-transmembrane domain model.

ACKNOWLEDGMENTS

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