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The Role of N-Linked Glycosylation in Protein Folding, Membrane Targeting, and Substrate Binding of Human Organic Anion Transporter hOAT4

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ABSTRACT

We used a novel approach to evaluate how the addition/acquisition and processing/modification of N-linked oligosaccharides play a role in the functional maturation of human organic anion transporter hOAT4. Inhibition of acquisition of oligosaccharides in hOAT4 by mutating asparagine to glutamine and by tunicamycin treatment was combined with the expression of wild-type hOAT4 in a series of mutant Chinese hamster ovary (CHO)-Lec cells defective in the different steps of glycosylation processing. We showed that both the disruption of the glycosylation sites by mutagenesis and the inhibition of glycosylation by tunicamycin treatment resulted in a nonglycosylated hOAT4, which was unable to target to the cell surface. In contrast, hOAT4 synthesized in mutant CHO-Lec cells, carrying different structural forms of sugar moieties (mannose-rich in Lec1 cells, sialic acid-deficient in Lec2 cells, and sialic acid/galactose-deficient in Lec8 cells) were able to traffic to the cell surface. However, hOAT4 expressed in CHO-Lec1 cells had significantly lower binding affinity for its substrates compared with that expressed in parental CHO cells. This study provided novel information that addition/acquisition of oligosaccharides but not the processing of the added oligosaccharides participates in the membrane insertion of hOAT4. Processing of added oligosaccharides from mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates. Glycosylation could therefore serve as a means to specifically regulate hOAT4 function in vivo.

Organic anion transporters (OATs) play essential roles in the body disposition of clinically important anionic drugs, including anti-human immunodeficiency virus therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (You, 2002). Several OAT isoforms have been cloned by us and others (You, 2004). OAT1 and OAT3 are expressed predominantly in the kidney and the brain. In the kidney, these transporters use 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 Na^+-dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is used by a dicarboxylate/organic anion (OA) exchanger to move the OA substrate into the cell. This cascade of events indirectly links OA transport to metabolic energy and the Na^+ gradient, allowing 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 (Ekaratanawong et al., 2004). OAT2 is predominantly expressed in the liver (You, 2002). The transport mechanism for OAT2 remains to be elucidated.

Computer modeling derived from hydropathy analysis predicted that these proteins have 12 putative membrane-spanning domains and multiple consensus sites for glycosylation and phosphorylation (You, 2002, 2004). In a previous study on the role of glycosylation in OAT1 function (Tanaka et al., 2004),

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ABBREVIATIONS: OAT, organic anion transporter; CHO, Chinese hamster ovary; OA, organic anion; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; endo, endoglycosidase; DHEA, dehydroepiandrosterone 3-sulfate; NHS-SS-biotin, biotin disulfide *N*-hydroxysuccinimide ester; h, human; m, mouse.

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 to glutamine and assessing the mutant transporters in HeLa cells. One of the findings from that study is that simultaneous replacement of all asparagines in both mOAT1 and hOAT1 resulted in the transporters trapped in the intracellular compartment, suggesting that glycosylation is essential for the targeting of the transporters to the plasma membrane. The possibility cannot be excluded, however, that the amino acid substitutions introduced in that study, per se, affected the membrane insertion of the transporters.

The processing of N-linked glycosylation of proteins occurs in several steps (Fig. 1) (Kornfeld and Kornfeld, 1985). First, a dolichol pyrophosphate precursor (Glc3Man9GlcNAc2) is added to asparagine side chain of asparagine-X-serine/threonine consensus sequence for N-linked oligosaccharides in a nascent polypeptide in the endoplasmic reticulum (acquisition of N-linked oligosaccharides). Processing then begins by the removal of the three terminal glucose residues and at least one mannose residue in the endoplasmic reticulum. The partially processed polypeptide is then transported to Golgi apparatus, in which mannose residues are further trimmed and *N*-acetylglucosamine, galactose, and sialic acid residues are sequentially added. The newly synthesized glycoproteins then exit the Golgi and are transported to their final destination.

In contrast to our previous study (Tanaka et al., 2004), which focused on the addition/acquisition step of the glycosylation through mutagenesis of N-linked glycosylation sites, the present study focused on the processing/modification steps of the glycosylation by expression of hOAT4 in a series of mutant CHO-Lec cells defect in different steps of glycosylation processing (Stanley and Siminovitch, 1977). CHO-Lec1 cells have no detectable *N*-acetylglucosaminyl-transferase I activity, and proteins expressed carry oligosaccharides bearing mannose-rich intermediates ($Man_5GlcNAc_2$) at sites normally occupied by complex carbohydrates ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$) in parental cell line CHO cells (Fig. 1). CHO-Lec2 and CHO-Lec8 cells lack CMP-sialic acid and UDP-galactose translocases, respectively, and are incapable of transporting CMP-sialic acid and UDP-galactose from the cytosol to the Golgi, thereby producing sialic acid-deficient ($Gal_2GlcNAc_2Man_3GlcNAc_2$, in CHO-Lec2 cells) and sialic acid-/galactose-deficient ($GlcNAc_2Man_3GlcNAc_2$, in CHO-Lec8 cells) complex oligosaccharides (Fig. 1).

Materials and Methods

[^{3}H]Estrone sulfate was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). Parental CHO-pro5 cell line and its mutant cell lines CHO-Lec1,

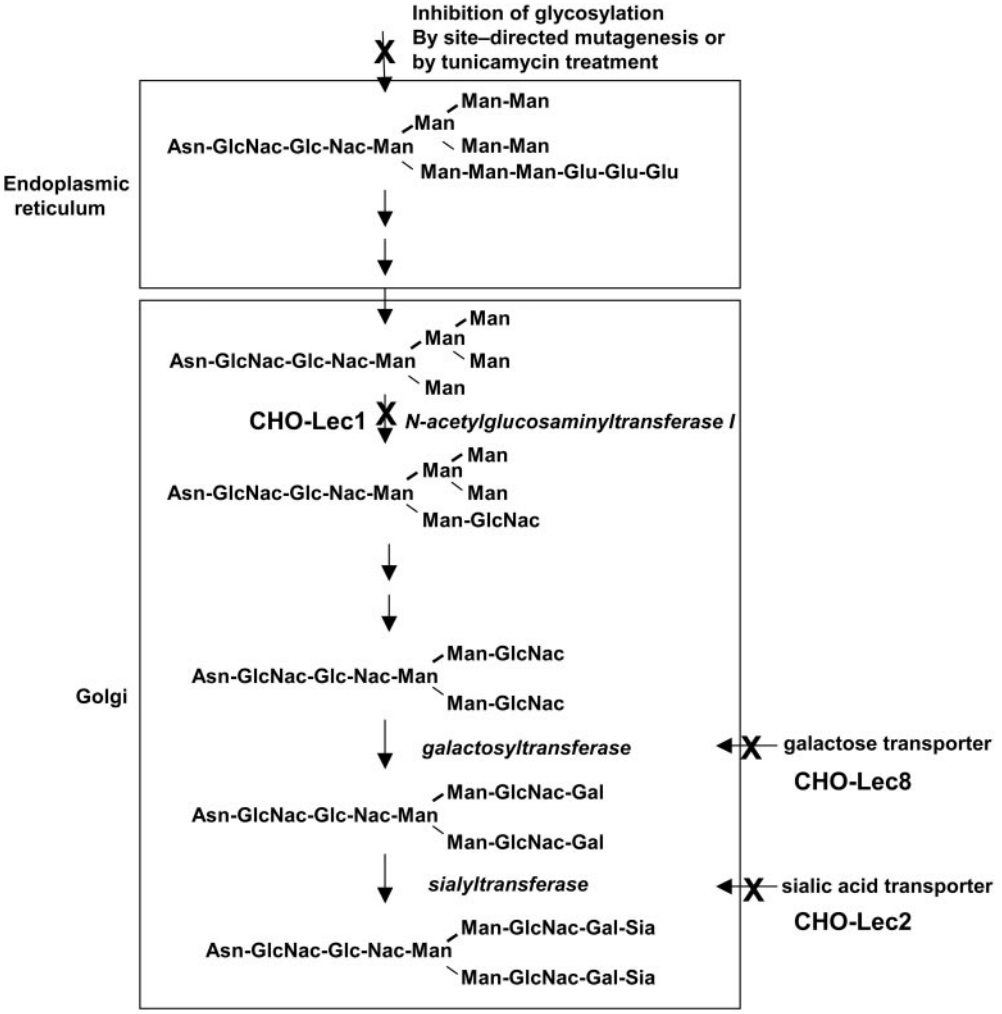

Fig. 1. Scheme of N-linked oligosaccharide biosynthetic pathway in the endoplasmic reticulum and the Golgi apparatus. The sites for disruption of glycosylation and defects in mutant CHO cells (CHO-Lec1, CHO-Lec2 and CHO-Lec8) are shown as X.

CHO-Lec2, and CHO-Lec8 were obtained from the American Type Culture Collection (Manassas, VA). The mutant CHO cells were originally established by Dr. Pamela Stanley (Stanley and Siminovitch, 1977). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Tunicamycin Treatment. The cells were treated with 10 μg/ml tunicamycin for 18 h. Tunicamycin inhibits transfer of dolichol pyrophosphate precursor to asparagine in the consensus sequence for N-linked glycosylation (asparagine-X-serine/threonine).

Site-Directed Mutagenesis. Mutant transporters were generated by site-directed mutagenesis of asparagine to glutamine in hOAT4. The mutant sequences were confirmed by the dideoxy chain-termination method.

Generation of Cells Stably Expressing hOAT4. Parental CHO-pro5 cells and its mutant cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8) were grown in minimal essential medium α supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO_2 atmosphere at 37°C. Cells were seeded at 3×10^6/100-mm dish 24 h before transfection. For transfection of hOAT4 cDNA plasmid, a LipofectAMINE 2000 reagent was used following the manufacturer's instruction. After 7 to 8 days of selection in medium containing 2 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), resistant colonies were replated to 96 wells for cloning, expansion, and analyzing positive clones.

Transport Measurement. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca^{2+}/Mg^{2+} (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3) and [^{3}H]estrone sulfate. At the times indicated in the figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. ($n = 3$).

Isolation of Plasma Membrane Proteins by Cell-Surface Biotinylation. Cell-surface expression level of hOAT4 was examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin. hOAT4 was expressed in Cos-7 cells or CHO cells in six-well plates. To initiate biotinylation, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS/CM, pH 8.0. The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 200 mg/ml protease inhibitor phenylmethylsulfonyl fluoride, and 3 mg/ml leupeptin, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Streptavidin-agarose beads (50 μl) were then added to the supernatant to isolate the cell-membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-hOAT4 antibody.

Deglycosylation of hOAT4 with Peptide *N*-Glycosidase F and Peptide *N*-Glycosidase H. For deglycosylation, proteins from total cell lystate were denatured in 1.5% SDS and 2.5% β-mercaptoethanol, heated at 50°C for 30 min, and then incubated in 50 mM sodium phosphate buffer, pH 7.5, 1% Nonidet P-40, and 1 μl of peptide *N*-glycosidase F (PNGase F, 500 units/μl; New England Biolabs, Beverly, MA) at 37°C for 1 h. The samples were then used for immunoblotting with anti-hOAT4 antibody.

Electrophoresis and Western Blot. Protein samples (with equal amount) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS/0.05% Tween, washed, and incubated for 2 h at room temperature with polyclonal anti-hOAT4 antibody (1:500). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000), and signals were detected by SuperSignal West Dura Extended Duration Substrate Kit (Pierce Chemical).

Immunofluorescence of Transfected Cells. Cells expressing hOAT4 were washed three times in phosphate-buffered saline, fixed for 20 min at room temperature in 4% paraformaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. The cells were incubated for 30 min at room temperature in PBS containing 5% goat serum and then incubated for 1 h in the same medium containing anti-hOAT4 antibody (1:500) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with fluorescein isothiocyanate-coupled goat anti-rabbit IgG (Chemicon International, Temecula, CA) diluted to 1:200 for 1 h. Treated cells were thoroughly washed, and the cover glasses were mounted in GEL/MOUNT (Biomeda, Foster City, CA). Samples were visualized with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY) or a regular fluorescence microscope.

Results

Analysis of the Role of Acquisition of Oligosaccharides in hOAT4 by Site-Directed Mutagenesis. Addition/acquisition of oligosaccharides is the first step in the glycosylation process. To determine its role in hOAT4 function, we disrupted the potential glycosylation sites by replacing asparagine with glutamine singly or in combination. We observed in Cos-7 cells that single replacement of asparagine with glutamine had no effect on cell-surface expression and transport function of hOAT4 (data not shown), suggesting that glycosylation at individual sites is not required for the transport function of hOAT4. When all of the glycosylation sites were simultaneously disrupted, the quadruple mutant protein (N39/N56/N63Q/N99Q) had a molecular mass of 47 kDa with or without the treatment of PNGase F (Fig. 2a), which removes sugar moieties from glycoproteins. This molecular mass is the same as that of nonglycosylated hOAT4, indicating that there are no additional sites for *N*-glycosylation. It is important to note that when all of the glycosylation sites were removed, the expression level of the nonglycosylated hOAT4 was almost diminished compared with that of wild-type hOAT4, although the total cellular protein of the quadruple mutant was similar to that of wild-type hOAT4 (Fig. 2, b and c). Immunofluorescence experiment (Fig. 2d) showed that the most of the quadruple mutant resided in the intracellular compartment. This result suggests that glycosylation is critical for the proper trafficking of the transporter onto the plasma membrane. Functional study (Fig. 2e) showed that there was virtually no transport activity in the quadruple mutant-transfected cells compared with that of wild-type hOAT4-transfected cells, consistent with their cellular distributions.

To determine whether the above observations were specific to Cos-7 cells, similar studies were performed in CHO cells. Again, despite the comparable total cell expression between wild-type hOAT4 and its quadruple mutant (Fig. 3, b and c), the cell-surface expression of the quadruple mutant was almost undetectable (Fig. 3, a and d). Therefore, the inability of the unglycosylated hOAT4 to target to the plasma membrane is an important feature of the transporter regardless of the cell type used for the studies. The quadruple mutant-trans-

fected cells were unable to transport [^{3}H]estrone sulfate (Fig. 3e), consistent with its cellular distribution in CHO cells.

Analysis of the Role of Acquisition of Oligosaccharides in hOAT4 by the Treatment of hOAT4-Expressing Cells with Tunicamycin. Because the possibility exists that the loss of the surface expression of nonglycosylated hOAT4 generated by replacing asparagine with glutamine (Figs. 2 and 3) may have resulted from amino acid substitutions per se rather than from deglycosylation of hOAT4, an additional experiment was performed by treating hOAT4-expressing cells with tunicamycin. Tunicamycin inhibits the first step of N-linked glycosylation (acquisition of N-linked oligosaccharides) without introducing amino acid substitution. Western blot analysis of plasma membrane proteins (Fig. 4a) showed that treatment of hOAT4-expressing cells with tunicamycin resulted in an almost complete loss of cell-surface expression of nonglycosylated hOAT4, despite that the total cell expression of nonglycosylated hOAT4 was similar to that of fully glycosylated hOAT4 (Fig. 4b). These results confirm that acquisition of oligosaccharides indeed plays an important role in the targeting of hOAT4 to the plasma membrane. Transport activity of hOAT4 in tunicamycin-treated cells was almost undetectable (Fig. 4c).

Western Blot Analysis of the Role of Processing of Oligosaccharides in hOAT4 Expressed in Mutant CHO-Lec Cells. Having demonstrated the role of acquisition of oligosaccharides in membrane insertion of hOAT4, we then switched our focus to the role of processing of oligosaccharides in hOAT4 function. Using clonal cell lines stably expressing the highest levels of hOAT4, we determined the molecular masses and the glycosylation patterns of hOAT4 expressed in parental CHO cells and the mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). In total cell lysates (Fig. 5), hOAT4 expressed in parental CHO cells had a molecular mass of 80 kDa. This protein was resistant to the treatment of endoglycosidase H (endo H) but sensitive to the treatment of endoglycosidase F (endo F). Endo H cleaves high mannose-containing immature N-linked carbohydrates from glycoproteins, whereas endo F cleaves both the high mannose-containing immature N-liked carbohydrates and complex-type oligosaccharides from glycoproteins. Treatment of the 80-kDa protein with endo F resulted in a reduction in its molecular size to that of nonglycosylated hOAT4. Therefore, hOAT4 expressed in parental CHO cells contained fully processed carbohydrates. hOAT4 expressed in CHO-Lec1 cells had a molecular mass of 50 kDa. This protein was sensitive to the treatment of both endo H and endo F. Treat-

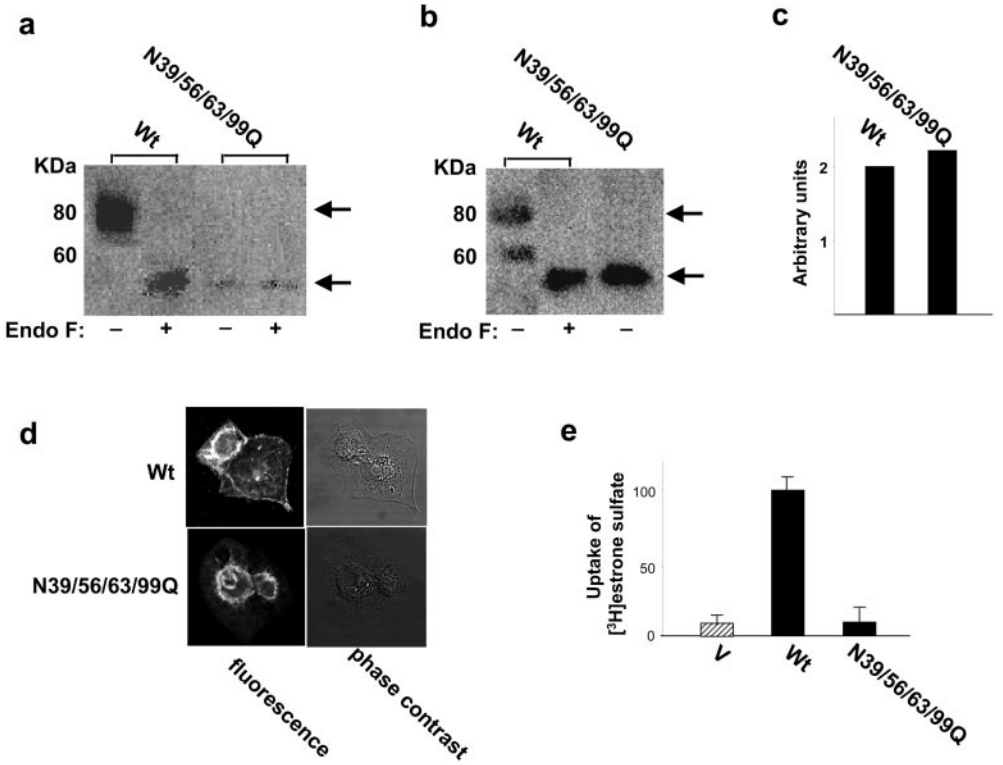

Fig. 2. The effect of disrupting glycosylation sites by mutagenesis on the surface expression, cellular distribution, and the function of hOAT4 in Cos-7 cells. a, cell-surface expression. Cos-7 cells transiently expressing wild-type (Wt) hOAT4 and its quadruple mutant (N39/56/63/99Q) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody. b, total cell expression. Cells transiently expressing Wt hOAT4 and its quadruple mutant were lysed, and their proteins were separated and visualized as described above. The ~80-kDa band represented the mature form of hOAT4, and the ~60-kDa band represented the immature form of hOAT4. Treatment of the total cell lysate from Wt hOAT4-transfected cells with endo F shifted both forms to nonglycosylated form (~47 kDa). c, quantification of the total cell expression of Wt hOAT4 and its quadruple mutant observed in Fig. 2b. The intensity of the sum for the ~80-kDa band and the ~60-kDa band in Wt hOAT4-transfected cells was similar to that of the ~47-kDa band in the quadruple mutant-transfected cells. d, immunolocalization. Cells transiently expressing Wt hOAT4 and its quadruple mutant were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions. e, [^{3}H]estrone sulfate uptake in Wt hOAT4 and its quadruple mutant-transfected cells. pcDNA vector (V)-transfected cells were used as mock control.

ment of the 50-kDa protein with both enzymes resulted in a reduction in its molecular size to that of nonglycosylated hOAT4. Therefore, hOAT4 expressed in CHO-Lec1 cells was a precursor form of hOAT4 in the endoplasmic reticulum. hOAT4 expressed in CHO-Lec2 and CHO-Lec8 cells had molecular sizes of 70 or 60 kDa, respectively. These proteins were resistant to the treatment of endo H but sensitive to the treatment of endo F, and therefore probably represented sialic acid-deficient (in CHO-Lec2 cells) and sialic acid/galactose-deficient (in CHO-Lec8 cells) complex-type glycoproteins. Biotinylation of cell-surface proteins with a membrane-impermeable reagent NHS-SS-biotin (Fig. 6) showed that

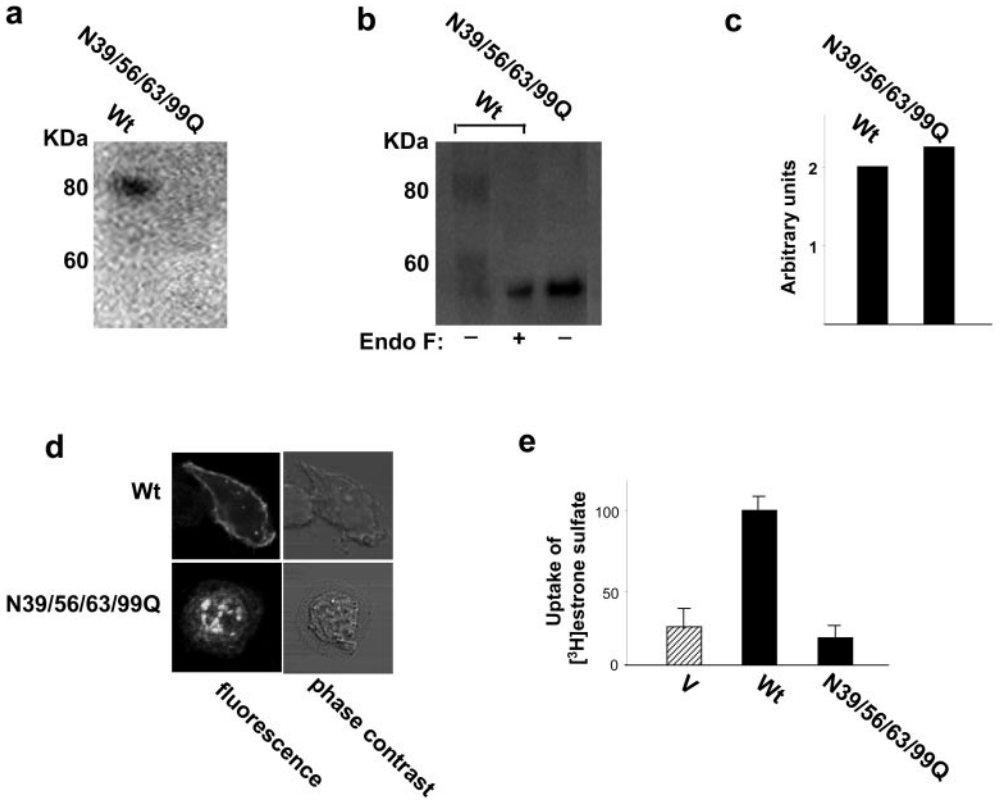

Fig. 3. The effect of disrupting glycosylation sites by mutagenesis on the surface expression, cellular distribution, and the function of hOAT4 in CHO cells. a, cell-surface expression. CHO cells transiently expressing wild-type (Wt) hOAT4 and its quadruple mutant (N39/56/63/99Q) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody. b, total cell expression. Cells transiently expressing Wt hOAT4 and its quadruple mutant were lysed, and their proteins were separated and visualized as above. The ~80-kDa band represented the mature form of hOAT4, and the ~60-kDa band represented the immature form of hOAT4. Treatment of the total cell lysate from Wt hOAT4-transfected cells with endo F shifted both forms to the nonglycosylated form, ~47 kDa. c, quantification of the total cell expression of Wt hOAT4 and its quadruple mutant observed in Fig. 3b. The intensity of the sum for the ~80-kDa band, the ~60-kDa band, and the ~47-kDa band in Wt hOAT4-transfected cells was similar to that of ~47-kDa band in the quadruple mutant-transfected cells. d, immunolocalization. CHO cells transiently expressing Wt hOAT4 and its quadruple mutant (N39/56/63/99Q) were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions. e, [^{3}H]estrone sulfate uptake in Wt hOAT4- and its quadruple mutant-transfected cells. pcDNA vector (V)-transfected cells were used as mock control.

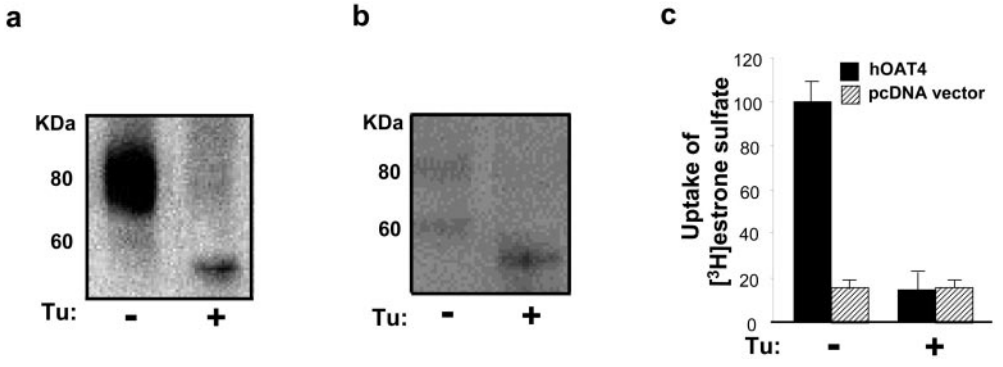

Fig. 4. The effect of inhibition of glycosylation by tunicamycin treatment on the cell-surface expression and the function of hOAT4. a, cell-surface expression. Cos-7 cells expressing wild-type (Wt) hOAT4 were treated with or without tunicamycin (Tu) followed by biotinylation. The labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody. b, total cell expression. Cells expressing Wt hOAT4 were treated with or without tunicamycin, lysed, and their proteins were separated and visualized as above. c, [^{3}H]estrone sulfate uptake in hOAT4-transfected cells treated with or without tunuicamycin. pcDNA vector (V)-transfected cells were used as mock control.

significant amount of hOAT4 expressed at the cell surface in both parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). Immunofluorescence study (Fig. 7) confirmed this observation. The amount of cell-surface expression of hOAT4 (Fig. 6) correlated with its total cell expression in these cells (Fig. 5).

Functional Analysis of the Role of Processing of Oligosaccharides in hOAT4 Expressed in Mutant CHO Cells. Transport of [^{3}H]estrone sulfate was then measured in hOAT4-expressing parental CHO cells and mutant CHO cells (Fig. 8). hOAT4 expressed in parental CHO cells, CHO-Lec2, and CHO-Lec8 exhibited transport activities that correlated with their surface expression (Fig. 6), whereas hOAT4 expressed in CHO-Lec1 exhibited much lower transport activity.

The functional properties of hOAT4 in theses cells were further characterized in several aspects. hOAT4 is known to function as an exchanger (3), with one organic anion being transported into the cells in exchange for another organic anion being effluxed out of the cells. To determine whether modification of oligosaccharides in hOAT4 expressed in mutant CHO cells affects this functional characteristic, hOAT4-expressing parental CHO cells and mutant CHO cells were preloaded with [^{3}H]estrone sulfate followed by exposing to medium with or without dehydroepiandrosterone 3-sulfate (DHEA), another substrate for hOAT4. As shown in Table 1, significant efflux of intracellular [^{3}H]estrone sulfate was observed with hOAT4 expressed in all types of cells when cells were exposed to medium containing an exchangeable substrate DHEA, whereas little efflux was observed with the medium lacking DHEA. hOAT4 expressed in mutant CHO cells also had substrate spectra similar to that in parental CHO cells (Fig. 9). These results suggest that processing/modification of oligosaccharides in mutant CHO cells had no significant effects on the basic property of hOAT4 as an organic anion exchanger and on its substrate spectra. Finally, kinetic analysis of estrone sulfate transport in hOAT4-expressing parental CHO cells and mutant CHO cells was performed. Table 2 showed an Eadie-Hofstee analysis of the derived data. hOAT4 expressed in CHO-Lec1 cells had much lower binding affinity (K_m = 16 ± 0.3 μM) compared with

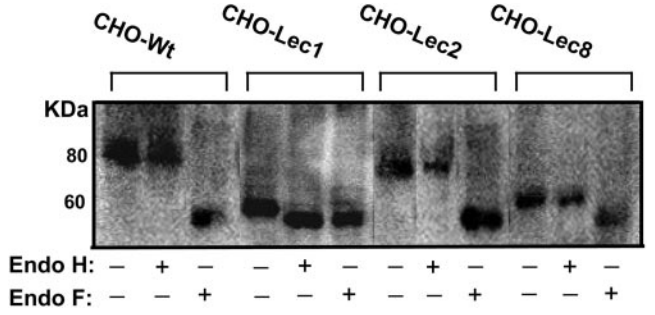

Fig. 5. Western blot analysis of the expression pattern of hOAT4 in total cell lysates from parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). Total cell lysate proteins isolated from hOAT4-expressing parental CHO cells and mutant CHO cells were treated with endo H or endo F, followed by Western blotting with anti-hOAT4 antibody.

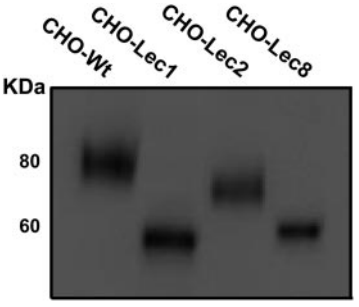

Fig. 6. Western blot analysis of cell-surface expression of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). Cells expressing hOAT4 were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody.

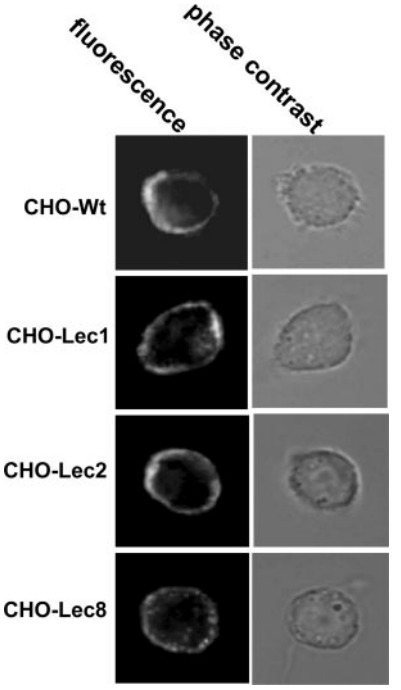

Fig. 7. Immunofluorescence of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). Cells expressing hOAT4 were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

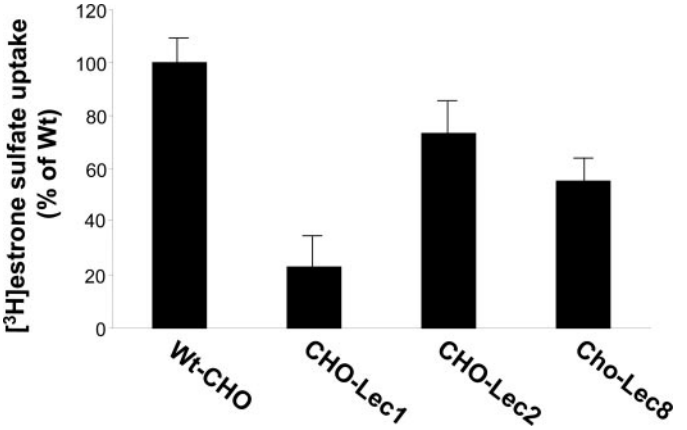

Fig. 8. [^{3}H]Estrone sulfate uptake into hOAT4-expressing parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). [^{3}H]Estrone sulfate uptake (100 nM and 5 min) was measured in hOAT4-expressing parental and mutant CHO cells. The data are presented as the percentage of uptake in parental CHO cells. Values given are mean ± S.E. (n = 3).

hOAT4 expressed in parental CHO cells (K_m = 6.0 ± 0.5 μM). The low binding affinity of hOAT4 in CHO-Lec1 cells may contribute to the low transport activity observed in these cells (Fig. 8).

Discussion

OATs play essential roles in the body disposition of clinically important anionic drugs, including antiviral drugs, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (You, 2002, 2004). hOAT4 and OAT1 have several distinct properties. OAT1 is localized in the basolateral membrane of the kidney proximal tubule cells and preferably transport small organic anions such as *para*-aminohippuric acid, whereas hOAT4 is localized in the apical membrane of the kidney proximal tubule cells and preferably transport bulkier organic anions such as estrone sulfate (You, 2002, 2004). One common structure feature shared among all the cloned OATs is the presence of consensus sites for N-linked glycosylation in the first extracellular loop within the current secondary structure model (You, 2002, 2004). We showed previously (Tanaka et al., 2004) that simultaneous replacement of all asparagines in OAT1 by mutagenesis impaired the trafficking of the transporter to the cell surface, suggesting an important role of glycosylation in the targeting of OAT1 onto the plasma membrane. In the present study, we showed that disruption of all the glycosylation sites in hOAT4 also rendered hOAT4 unable to traffic to the cell surface (Figs. 2 and 3). Therefore, the role of glycosylation in membrane insertion of the transporters could be a common characteristic for all members of the OAT family. However, the results obtained from the above-mentioned experiments cannot rule out the possibility that the loss of the surface expression of nonglycosylated OATs (OAT1 in the previous study and hOAT4 in the present study) gen-

TABLE 1

Efflux of [^{3}H]estrone sulfate mediated by hOAT4 expressed in wild-type CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8)

Cells expressing hOAT4 were preloaded with [^{3}H]estrone sulfate (500 nM) for 1 h, followed by exposure to phosphate-buffered saline (control group) or phosphate-buffered saline containing unlabeled DHEA (experimental group). The amount of intracellular [^{3}H]estrone sulfate in the experimental group was expressed as a percentage of that in the control group. The results shown are means ± S.E. (n = 3).

Mutants	Intracellular Remaining [^{3}H]Estrone Sulfate
	% of Control
CHO-Wt	52.4 ± 4.0
CHO-Lec1	57.0 ± 6.4
CHO-Lec2	53.1 ± 7.4
CHO-Lec8	59.0 ± 4.0

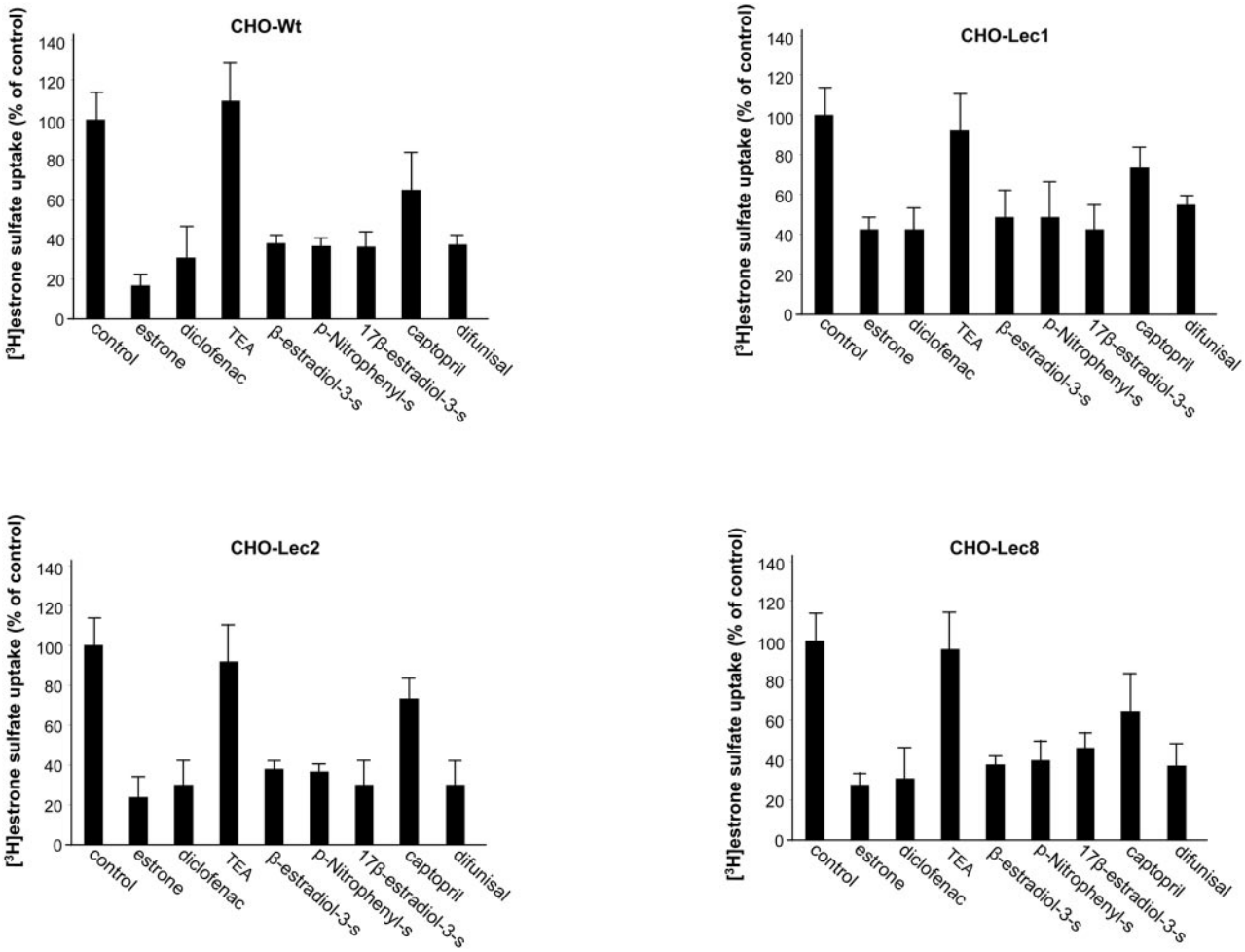

Fig. 9. Substrate selectivity of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). The substrate selectivity was obtained in competition experiments. Uptake of [^{3}H]estrone sulfate (100 nM) was measured in the absence (control) and presence of various unlabeled compounds (1 μM). The data are presented as the percentage of control uptake. Values given are mean ± S.E. (n = 3).

TABLE 2

Kinetic analysis of hOAT4-mediated estrone sulfate transport in wild-type CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8)

Kinetic characteristics were determined at substrate concentrations ranging from 0.05 to 10 μM (5-min uptake) in hOAT4-expressing wild-type CHO cells and mutant CHO cells. Transport kinetic values were calculated using the Eadie-Hofstee transformation. Values given are mean ± S.E. (n = 3).

Cell Lines	K_m
	μM
CHO-Wt	6.0 ± 0.5
Lec1	16.0 ± 0.3
Lec2	4.5 ± 0.6
Lec8	4.6 ± 0.45

erated by mutagenesis may result from amino acid substitutions per se rather than from deglycosylation of transporters. To address this issue, we performed a new experiment in the present study by treating hOAT4-expressing cells with tunicamycin. Tunicamycin inhibits the first step of N-linked glycosylation (acquisition of N-linked oligosaccharides) without introducing amino acid substitution. We showed that the nonglycosylated hOAT4 in tunicamycin-treated cells was unable to traffic to the cell surface, confirming that acquisition of oligosaccharides indeed play a critical role in the targeting of the transporters to the cell surface. Several roles of the addition of N-linked oligosaccharides to nascent proteins have been demonstrated; for example, N-linked oligosaccharides attach to lectin-like molecular chaperons such as calnexin and calreticulin, facilitating correct protein folding, and also play a role in the "quality control" system of the endoplasmic reticulum that ensures selective transportation of the properly folded proteins for the Golgi complex (Helenius, 1994). Therefore, unfolded proteins such as nonglycosylated hOAT4 produced by site-directed mutagenesis or by tunicamycin treatment may have been trapped in the endoplasmic reticulum and was unable to target to the cell surface. The role of glycosylation in membrane insertion has been observed for proteins such as ATP-sensitive potassium channel (Conti et al., 2002), glycine transporter GLYT1 (Olivares et al., 1995), and gastric H,K-ATPase (Vagin et al., 2004). However, glycosylation is not important for proteins such as serotonin transporter (Tate and Blakely, 1994), and the sodium-dependent, purine-selective nucleoside transporter SPNT (Mangravite and Giacomini, 2003).

In contrast to our previous study (Tanaka et al., 2004) that focused on the acquisition step of the glycosylation in hOAT1 using a mutagenesis approach, the present study focused on the processing steps of the glycosylation in hOAT4 by expressing the transporter in a series of mutant CHO-Lec cells deficient in different steps of glycosylation processing. Lec1 cells lack *N*-acetylgalactosaminyl-transferase I and hence give rise to oligosaccharides bearing high mannose intermediates. CHO-Lec2 and CHO-Lec8 cells lack CMP-sialic acid and UDP-galactose translocases and are incapable of transporting CMP-sialic acid and UDP-galactose from the cytosol to the Golgi, thereby producing sialic acid-deficient (in CHO-Lec2 cells) and sialic acid-/galactose-deficient (in CHO-Lec8 cells) complex oligosaccharides, respectively (Fig. 1). To our knowledge, such a new approach has not been used for investigating the processing/modification of oligosaccharides in any of the drug transporters cloned so far.

It is interesting that our biotinylation experiments (Fig. 6), in conjunction with immunofluorescence studies (Fig. 7), showed that hOAT4, whether containing high mannose oligosaccharides (in CHO-Lec1 cells), sailic acid-deficient oligosaccharides (in CHO-Lec2 cells), or sailic acid-/galactose-deficient oligosaccharides (in CHO-Lec8 cells), was efficiently expressed at the cell surface, suggesting that once the acquisition of oligosaccharides is accomplished, the conformational maturation of the transporter is achieved, which leads to the targeting of the transporter onto the plasma membrane. The targeting of the transporter to the cell surface may not depend on the processing of the added oligosaccharides, whereas nonglycosylated hOAT4 generated by site-directed mutagenesis and by tunicamycin treatment may not be folded correctly and was unable to traffic to the cell surface.

We also showed that hOAT4 terminating with different types of N-linked oligosaccharides (high mannose type, sialic acid-deficient type, or sialic acid-/galactose-deficient type) preserved the basic functional properties of wild-type hOAT4 as an organic anion exchanger (Table 1). hOAT4 terminating with different types of N-linked oligosaccharides also had substrate spectra similar to those of wild-type hOAT4 (Fig. 9). Therefore, processing of the added oligosaccharides may not be critical in determining these functional properties.

However, the processing of the added oligosaccharides did affect the binding affinity of hOAT4 for its substrates. Our kinetic analysis (Table 2) revealed that hOAT4 terminating with high mannose oligosaccharides (in CHO-Lec1 cells) had significantly lower binding affinity for its substrates compared with that of hOAT4 expressed in parental CHO cells, suggesting that processing of added oligosaccharides from high mannose type to complex type strongly enhanced the ability of hOAT4 to bind its substrates, although the binding affinity may not be necessarily dependent on the completion of complex oligosaccharides, because hOAT4 with sialic acid-deficient oligosaccharides (in CHO-Lec2 cells) and sialic acid-/galactose-deficient oligosaccharides (in CHO-Lec8 cells) has binding affinities similar to those of hOAT4 expressed in parental CHO cells. On the molecular level, the presence of sialic acid and galactose at the terminus of the oligosaccharides may not contribute to the binding affinity of hOAT4 for its substrates. Rather, the *N*-acetylglucosamine, which is present in the oligosaccharides synthesized from the parental CHO cells and from mutant cells Lec2 and Lec8 but is missing in the oligosaccharides synthesized from Lec1 cells, may play a role in the binding affinity of hOAT4 for its substrates. The 2-acetamide group in *N*-acetylglucosamine has a different orientation compared with the 2-hydroxyl group in mannose (from Lec1 cells) despite that the rest of the structures are quite similar between *N*-acetylglucosamine and mannose. Therefore, it is possible that the structure of *N*-acetylglucosamine favors the binding of hOAT4 substrates over that of mannose. Our data on hOAT4 may not be generalized to other proteins because the signaling activity for intercellular adhesion molecule 1 (Otto et al., 2004) was reduced in all of the CHO-Lec cells, and the cell-surface expression levels of thyrotropin receptor TSHR were decreased in both Lec1 and Lec2 cells (Nagayama et al., 1998).

In conclusion, the present study provided novel information, which was not addressed in our previous study (Tanaka et al., 2004), that the addition/acquisition of oligosaccharides but not the processing of the added oligosaccharides plays a

critical role in the targeting of hOAT4 to the plasma membrane. Processing of added oligosaccharides may not be essential in determining the substrate spectra of hOAT4 and its property as an organic anion exchanger. However, the processing of added oligosaccharides form mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates. Glycosylation may be altered depending on the cell type and physiological states and therefore could serve as a means to specifically regulate hOAT4 function in vivo.

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