Heterologous Expression and Functional Characterization of a Mouse Renal Organic Anion Transporter in Mammalian Cells*

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Organic anion transporters play an essential role in eliminating a wide range of organic anions including endogenous compounds, xenobiotics, and their metabolites from kidney, thereby preventing their potentially toxic effects within the body. The goal of this study was to extend our previous study on the functional characterization and post-translational modification of a mouse kidney organic anion transporter (mOAT), in a mammalian cell system, COS-7 cells. The transporter-mediated p-aminohippurate (PAH) uptake was saturable, probenecid-sensitive, and inhibited by a wide range of organic anions including vitamins, anti-hypertensive drugs, anti-tumor drugs, and anti-inflammatory drugs. Tunicamycin, an inhibitor of asparagine-linked glycosylation, significantly inhibited the transport activity. Immunofluorescence provided evidence that most of the protein remained in the intracellular compartment in tunicamycin-treated cells. Diethyl pyrocarbonate (DEPC), a histidine residue-specific reagent, completely blocked PAH transport. The inhibitory effect by DEPC was significantly protected (90%) by pretreating the cells with excess unlabeled PAH, suggesting that the histidine residues may be close to the PAH binding sites. Finally, in situ mRNA localization was studied in postnatal mouse kidney. The expression was observed in proximal tubules throughout development. We conclude that COS-7 cells may be useful in pharmacological and molecular biological studies of this carrier. The carbohydrate moieties are necessary for the proper trafficking of mOAT to the plasma membrane, and histidine residues appear to be important for the transport function.

Renal organic anion transport plays a vital role in the elimination of a wide variety of potentially toxic, negatively charged waste products of metabolism, drugs, environmental pollutants, and their metabolites from the body. The transport mechanisms responsible for this elimination have been studied extensively (1–3). Based on these studies, it has been suggested that the transport of organic anions is a complex process involving distinctly different proteins at the apical and basolateral membranes of the proximal tubule cells. Organic anions are transported across the basolateral membrane into the cell in exchange for intracellular dicarboxylates, which are subsequently returned into the cell via a sodium-dependent dicarboxylate transporter. The luminal exit of organic anions is thought to occur by anion exchange and/or facilitated diffusion (1–3).

We previously reported the cloning of a mouse cDNA (termed NKT) that encodes a 546-amino acid membrane protein specifically expressed in the kidney (7). Subsequently, its counterparts from rat and winter flounder were isolated independently by other groups (4–6). Although expression studies in Xenopus oocytes revealed that this membrane protein had the characteristics of renal organic anion transporter (4–6), little is known concerning the structural basis for the function of this transporter. The availability of the cDNA that encodes the organic anion transporter protein enables us to begin to unravel the structure-function relationship of this elimination system at the molecular level.

We predicted in our early study (7) that the extracellular loop between putative transmembrane regions 1 and 2 contains four potential N-linked glycosylation consensus sites at positions Asn-56, Asn-86, Asn-91, and Asn-107. The same profile was also observed in its isoforms from rat and winter flounder (4–6). The presence of conserved N-glycosylation sites in all the organic anion transporters cloned so far suggests that glycosylation may play an important role in the function of these proteins.

A study using brush-border membrane vesicles from dog kidney (8) implied that histidine residues may be important for organic anion transport. To elucidate the diversity of the substrate recognition by the organic anion transporters, a signal feature of the organic anion transport system, it is important to clarify the mechanisms involved in the interaction of the substrates with the essential residues such as histidine residues of mOAT.1

Because it is highly desirable to express mammalian transport proteins in a mammalian expression system to ensure the appropriate post-translational processing, in the present study we have pursued an alternative expression system in a mammalian cell line, COS-7 (9). We showed that, when transfected with mOAT cDNA, the COS-7 cell system is functionally active as an organic anion transporter, and it exhibits similar general characteristics as described previously in other systems (4–6, 10). We now show that PAH transport by mOAT can be inhibited by vitamins and a variety of anionic drugs. Here we provide the first evidence that glycosylation of mOAT is necessary for the proper trafficking of the transporter onto the plasma membrane. We also suggest that histidine residues may be important for the transport function. To evaluate the role of mOAT during kidney development, we have studied the expression of mOAT in the developing mouse kidney.

EXPERIMENTAL PROCEDURES

DNA Constructs—Full-length cDNAs encoding mOAT with and without epitope tags, were subcloned into the mammalian expression vector pcDNA3.1(–) (Invitrogen). The epitope-tagged constructs encoded a

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1 The abbreviations used are: mOAT, mouse kidney organic anion transporter; PAH, p-aminohippurate; PBS, phosphate-buffered saline; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol.
fusion protein consisting of full-length mOAT with 10 amino acids of the human c-Myc epitope (EQKLISEEDL, nucleotide GAACAAAAGCT-GATTTCTGAAGAAGACCTG) at the carboxyl terminus. Tagged mOAT cDNA was synthesized by the polymerase chain reaction amplification. The polymerase chain reaction product was subcloned into plasmid pcDNA3.1 at XbaI and HindIII sites. The sequence was confirmed by the dideoxy chain termination method.

**Expression in COS-7 Cells**—COS-7 cells were grown in 24-well plates (50–80% confluency) at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were transfected with either 0.25 μg/well of pcDNA3.1(-)-mOAT, pcDNA3.1(-)-mOAT-myc, or pcDNA3.1(-) using LipofectAmine reagent (Life Technologies, Inc.) following the manufacturer’s instruction. The transfection efficiency was ~30% in all experiments as estimated by visualization by fluorescence microscopy after staining of mOAT-myc-transfected cells using anti-myc antibody in conjunction with fluorescein isothiocyanate-conjugated secondary antibody. The cells were used for transport measurements or immunostaining 48–72 h after transfection.

**Transport Measurements**—For each well, uptake solution (140 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 5 mM Tris, pH 7, and 20 μM ¹⁴C-labeled PAH) was added. At times indicated in the figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the plate with ice-cold washing solution (140 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, and 5 mM Tris, pH 7). The cells were solubilized in 0.2 N NaOH and aliquoted for liquid scintillation counting. The protein concentration was determined using the Bradford dye-binding procedure (11). For the inhibition studies, PAH uptake was measured in the presence of 0.3 mM to ≥1 mM substrates as indicated.

**In Vitro Transcription and Translation**—cRNA was synthesized from various pcDNA3.1(-) subclones using an in vitro transcription kit (Stratagene). The cRNAs were then translated in a rabbit reticulocyte lysate system (Promega) with L-[³⁵S]methionine (NEN Life Science Products) as described earlier (12). Canine pancreatic microsomes (Promega) were added to some of the translation reactions, which were then centrifuged at 4° C for 30 min in buffer containing 2.5% glycerol. SDS sample buffer (125 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 8 M urea, 20% sucrose, 0.5 mg/ml bromphenol blue) was added to all of the translation reactions, which were then boiled for 5 min before SDS-polyacrylamide gel electrophoresis.

**Immunofluorescence of Transfected Cells**—72 h after transfection, COS-7 cells were washed three times in phosphate-buffered saline (PBS), fixed for 15 min at room temperature in 4% paraformaldehyde (PFA), and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. After that, the cells were incubated for 15 min at room temperature in PBS containing 5% goat serum and then incubated for 1 h in the same medium containing anti-myc antibody (1:100) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Chemicon) diluted 1:100 for 1 h. Cells were thoroughly washed, and the cover glasses were mounted in 90% glycerol plus Citifluor. Samples were visualized on a fluorescence microscope.
microscope.

In Situ Hybridization—Developing mouse kidneys were collected, rinsed in PBS, and then fixed in ice-cold freshly prepared 4% paraformaldehyde/PBS for 1 h. They were then rinsed in 0.9% NaCl and dehydrated through a graded series of ethanol and embedded in paraffin. 7-μm sections were cut, mounted on slides, dewaxed, pretreated, and prehybridized as described previously (7). Antisense RNA probes labeled with [α-35S]UTP (Amersham) were produced. Hybridization was done overnight at 50 °C. Post-hybridization treatments were as follows: (i) two washes in 50% formamide, 2× SSC, 20 mM 2-mercaptoethanol (FSM) at 60 °C for 30 min, (ii) digestion with 10 μg/ml RNase A in 4× SSC, 20 mM Tri-HCl (pH 7.6), 1 mM EDTA at 37 °C for 30 min, and (iii) two washes in FSM at 60 °C for 45 min. Slides were dipped in Kodak NTB-2 emulsion and exposed for 10 days, and photographs were taken using a Leica DMRB microscope.

RESULTS

Kinetics of PAH Transport—Our preliminary experiment showed that pcDNA3.1(−)mOAT-transfected cells gave a linear uptake of 14C-labeled PAH for 1 h (data not shown). The kinetics of PAH transport were studied using 10-min uptake points. The initial rate of PAH uptake over a wide range of PAH concentrations was determined (Fig. 1a). Consistent with the previously reported data (4–6, 10), the transport of PAH across the cell membrane was saturable (Fig. 1b). Based on Eadie-Hofstee plot analysis (Fig. 1b, inset), the K_m value for PAH was 37.3 μm and V_max was 210 pmol/mg/10 min.

Substrate Specificity—We examined the substrate specificity of the carrier in competition experiments. A representative experiment of the relative inhibition of PAH (20 μm) uptake by 0.3 mM to −1 mM substrates is shown in Fig. 2. Because kidney is typically more efficient in the elimination of water-soluble compounds than liver, which is more efficient in the biliary disposal of liposoluble compounds (13), we tested the inhibition effect of water-soluble vitamins on mOAT-mediated PAH transport. Among the vitamins examined, riboflavin inhibited the transport activity with deglycosylation. Our strategy was to engineer an epitope tag (c-myc) to the carboxyl terminus of the protein so that the expressed protein could be detected using an anti-myc monoclonal antibody. To ensure that the epitope tag had little effect on the transport function, we measured the [14C]PAH uptake into both mOAT and mOAT-myc-transfected cells. As shown in Fig. 4, both mOAT and mOAT-myc showed similar transport activity and were equally sensitive to the general inhibitor of glycosylation, tunicamycin.

The Role of Glycosylation in the Transport Function—In our previous study (7), we reported the presence of four potential glycosylation sites located in the first extracellular loop between transmembrane domains 1 and 2 of mOAT. To determine whether carbohydrate moieties play a role in the function of the protein, we carried out initial experiments using the general inhibitor of glycosylation, tunicamycin. When COS-7 cells transfected with pcDNA3.1(−)mOAT were pretreated with 10 μg/ml tunicamycin for 24 h, an almost complete inhibition in the transport activity was observed (Fig. 3a). The potential use of these glycosylation sites was supported by our in vitro translation data of mOAT cRNA using a rabbit reticulocyte lysate system followed by 7.5% SDS-polyacrylamide gel electrophoresis (Fig. 3b). The major mOAT translation product synthesized in the presence of microsomes is larger than the ~58-kDa product synthesized in the absence of microsomes. The glycosome-dependent shift in the size of the mOAT translation product was reversed by endoglycosidase H treatment, indicating that the decreased mobility observed after translation in the presence of microsomes is due to glycosylation.

Next we investigated the reasons for a decrease in the transport activity with deglycosylation. Our strategy was to engineer an epitope tag (c-myc) to the carboxyl terminus of the protein so that the expressed protein could be detected using an anti-myc monoclonal antibody. To ensure that the epitope tag had little effect on the transport function, we measured the [14C]PAH uptake into both mOAT and mOAT-myc-transfected cells. As shown in Fig. 4, both mOAT and mOAT-myc showed similar transport activity and were equally sensitive to the inhibitor, probenecid. The transport activities of both constructs were blocked to the same extent by treatment of 10

![Fig. 3. The effect of glycosylation on the transport function.](image)

![Fig. 4. Functional comparison of mOAT and its myc-tagged product.](image)
μg/ml tunicamycin, suggesting that the myc-tagged construct retains the functional properties of the native (unmodified) structure.

Experiments were next carried out to examine the protein expression pattern of mOAT-myc before and after the treatment with tunicamycin using immunofluorescence. We showed that the plasma membrane was clearly labeled in the mOAT-transfected cells (Fig. 5a) as compared with control cells (Fig. 5e). In contrast, fluorescence remained mainly in the intracellular compartment after tunicamycin treatment (Fig. 5c). The right panel (Fig. 5, b, d, and f) shows that cells were fully attached to the culture dishes under all conditions, suggesting that the level of tunicamycin used was not toxic to the cells.

The Role of Histidine Residues in the Transport Function—A previous study using brush-border membrane vesicles from dog kidney (8) implied that histidine residues may be important for organic anion transport. Therefore, the effect of the histidyl modifier, DEPC, on PAH transport was examined. Fig. 6a shows that 1 mM DEPC completely blocked mOAT-mediated PAH transport, and the presence of 100 μM unlabeled PAH is capable of providing significant protection (90%) against the inhibitory effect by DEPC.

The specificity of DEPC was further investigated by examining transport after washing with a sulfhydryl restoring agent, dithiothreitol (DTT). If the sulfhydryl groups were modified by DEPC, then washing with DTT would result in restoration (14). As shown in Fig. 6a, treatment with DTT (10 mM) did not restore PAH transport after modification by DEPC.

It is conceivable that tyrosine residues could have been modified by DEPC under the conditions employed (15). Therefore, another approach to identify the group modified by DEPC was to examine what effect the tyrosine-directed reagent N-acety-
limidazole had on PAH transport. As shown in Fig. 6b, N-acetylimidazole (1 mM) had no effect on PAH transport. In Situ mRNA Localization of mOAT in Developing Mouse Kidneys—To study the maturation of the organic anion transport system in the developing kidney, mRNA localization in the kidneys of 1-day- and 1-week-old mice were studied using sense and antisense cRNA, and the data were compared with that of adult kidney. Fig. 7 showed that the most intense signal was present in kidney cortex, following a pattern characteristic of proximal tubular localization. There was no detectable signal in the glomeruli (arrows), distal tubules, or medulla (G). This mRNA expression pattern was obvious at the time of birth and continued throughout development.

DISCUSSION

The goal of this study was to extend our previous study on the functional characterization of mOAT and identification of elements important for its function. In this study, mOAT-transfected COS-7 cells were shown to be a valid model system for the functional analyses. The affinity ($K_m$) for the protosubstrate PAH in COS-7 cells (~37.3 µM) was comparable with the $K_m$ obtained from other systems (14–80 µM) (4–6, 10). It has been suggested that nephrotoxicity may be related to the elimination of certain drugs by organic anion transport system (16, 17). Therefore we examined a series of anionic drugs that may cause renal dysfunction (18) by examining their inhibitory effect on mOAT-mediated PAH transport. Our result showed that nonsteroidal anti-inflammatory drugs, indomethacin, sulindac, diclofenac, and carprofen almost completely blocked PAH transport. Anti-hypertensive drugs captopril and enalapril and anti-tumor drugs methotrexate and semustine showed moderate inhibition suggesting that renal clearance of these drugs may be through the organic anion transport system.

Our previous study predicted that four potential N-linked glycosylation sites were located in an extracellular loop between transmembrane domains 1 and 2. Glycosylation of one or more of these sites was suggested by our in vitro translation study (Fig. 3b). Furthermore, as demonstrated by the effect of tunicamycin on PAH uptake (Fig. 3e), N-linked asparagine glycosylation appeared necessary for functional expression of the transporter. It has been suggested that the addition of oligosaccharides to proteins is an integral step in the proper sorting, translocation, and insertion of polypeptides into membranes (19). Glycosylation may also promote resistance to proteolytic attack (20). Therefore, tunicamycin, which is known to prevent the initial step in the glycosylation pathway, could potentially affect transport proteins by several mechanisms. Among the various possibilities, our immunofluorescence study suggested that glycosylation was necessary for proper targeting of mOAT to the plasma membrane. Modifications of the oligosaccharide structure of glycoproteins related to changes in the biological activity of transporter proteins have been described. The GLUT1 glucose transporter (21, 22), the organic cation transporter of renal brush-border membrane (23), are examples of such a correlation. However, glycosylation is not required for the transport activity of the serotonin transporter.
Characteristics of mOAT: Drug Interactions and Structure/Function

(24). Here we provide the first evidence functionally, biochemically, and morphologically that glycosylation of mOAT is essential for the transport function.

In our present study, we also examined the possible involvement of the cationic amino acid, histidine, in the normal function of the PAH transport carrier. Other studies have shown that histidine groups are involved in the normal function of a number of membrane transporters (8, 25). The results from our studies with the histidine-specific reagent DEPC showed that this reagent caused a significant inhibition in the initial rate of PAH transport (Fig. 6a). DEPC is considered to be a specific histidine reagent when used at a pH range of 5.5 to 7.5. At higher pH values, DEPC specificity, however, decreases, and the compound begins to interact with other groups such as thiol groups and tyrosine residues. Because our experiment was conducted at pH 7.0, it was reasonable to assume that DEPC was interacting with histidine residues in the PAH transporter. This conclusion was further supported by the finding that DTT was unable to restore DEPC-induced inactivation, and N-acetylimidazole, a highly specific tyrosine-modifying reagent, was incapable of inhibiting PAH transport. This argues against the possibility that DEPC was interacting with thiol groups and tyrosine residues (Fig. 6). Our result showing that the presence of substrate protected the transporter from DEPC inactivation suggested that histidine residue(s) may be close to the PAH binding sites. Further studies using site-directed mutagenesis of mOAT will provide additional information regarding the roles of histidine residues in substrate recognition by the organic anion transporters.

Finally, early investigations suggested that the ability of the newborn kidney to eliminate organic anions is limited in comparison with the ability of an adult kidney (27, 28). Our in situ hybridization study shows that mOAT mRNA was expressed in proximal tubules at the time of birth, and the same pattern was observed throughout development. The correlation of the transport activity and the mRNA expression needs to be examined further.

In summary, our present study suggests that pcDNA3.1(−) mOAT-transfected COS-7 cells can serve as an in vitro model system for studying the pharmacology and molecular biology of the cloned mOAT. Many drugs that can cause renal dysfunction, such as anti-tumor drugs, antibiotics, and nonsteroidal anti-inflammatory drugs, are transported by the organic anion transport system. Therefore, our in vitro model system for screening drugs will facilitate the elucidation of the molecular basis of drug-related nephrotoxicity. Our study also presents data characterizing the impact of N-glycosylation and histidine residue(s) on the function of the cloned mOAT. The results contribute to our understanding of the relationship between structure and function of the organic anion transporters.

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