Human organic anion transporter 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-inflammatory drugs. To gain insight into the regulation of hOAT1, detailed information on its structural assembly is essential. In the present study, we investigated the quaternary structure of hOAT1 using combined approaches of chemical cross-linking, gel filtration chromatography, co-immunoprecipitation, cell surface biotinylation, and metabolic labeling. Chemical cross-linking of intact membrane proteins from LLC-PK1 cells stably expressing hOAT1 converted quantitatively hOAT1 monomer to putative trimer and higher order of oligomer, indicating that hOAT1 is present in the membrane as multimeric complexes. When co-expressed in LLC-PK1 cells, FLAG-tagged hOAT1 co-immunoprecipitated with myc-tagged hOAT1. The hOAT1 oligomer was also detected in gel filtration chromatography of total membrane proteins from hOAT1-expressing LLC-PK1 cells. Cell surface biotinylation with membrane-permeable reagents and metabolic labeling with [35S]methionine followed by immunoprecipitation showed that the oligomeric hOAT1 did not contain any other proteins. Taken together, this is the first study demonstrating that hOAT1 exists in the plasma membrane as a homooligomer, possibly trimer, and higher order of oligomer.

Organic anion transporters (OATs) 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-inflammatory drugs. To gain insight into the regulation of hOAT1, detailed information on its structural assembly is essential. In the present study, we investigated the quaternary structure of hOAT1 using combined approaches of chemical cross-linking, gel filtration chromatography, co-immunoprecipitation, cell surface biotinylation, and metabolic labeling. Chemical cross-linking of intact membrane proteins from LLC-PK1 cells stably expressing hOAT1 converted quantitatively hOAT1 monomer to putative trimer and higher order of oligomer, indicating that hOAT1 is present in the membrane as multimeric complexes. When co-expressed in LLC-PK1 cells, FLAG-tagged hOAT1 co-immunoprecipitated with myc-tagged hOAT1. The hOAT1 oligomer was also detected in gel filtration chromatography of total membranes from hOAT1-expressing LLC-PK1 cells. Cell surface biotinylation with membrane-permeable reagents and metabolic labeling with [35S]methionine followed by immunoprecipitation showed that the oligomeric hOAT1 did not contain any other proteins. Taken together, this is the first study demonstrating that hOAT1 exists in the plasma membrane as a homooligomer, possibly trimer, and higher order of oligomer.

**MATERIALS AND METHODS**

[14C]p-Aminohippuric acid (PAH) was from PerkinElmer Life Sciences. Membrane-impermeable biotinylation reagents NHS-SS-biotin, sulfo-NHS-LC-biotin, and biotin hydrazide, cross-linking reagents BS3, DSS, and DTSSP, and streptavidin-agarose beads were purchased from Pierce. PFO was purchased from Oakwood Products (West Columbia, SC). Protein A-agarose beads were purchased from Invitrogen. Superdex 200 column and molecular weight markers blue dextran, apoferritin, β-amylase, bovine serum albumin, ovalbumin, and carbonic anhydrase were from Amersham Biosciences. LLC-PK1 cells were purchased from American Type Culture Collection (Manassas, VA). All other reagents were purchased from Sigma.

**Construction of Epitope-tagged Transporters**—To facilitate immunodetection of hOAT1, epitope tag FLAG or myc was added to hOAT1 by site-directed mutagenesis, respectively. FLAG tag was also added to rat sulfate-anion antiporter rSat-1 (Open Biosystems, Huntsville, AL). The mutant sequences were confirmed by the dideoxy chain termination method.

**Generation of LLC-PK1 Cells Stably Expressing hOAT1-myc**—LLC-PK1 cells were grown in Medium 199, supplemented with 10% fetal calf serum.
HOAT1 Forms Homooligomers

serum, penicillin/streptomycin (100 units/ml) in a 5% CO₂ atmosphere at 37 °C. Cells were seeded at 3 × 10⁶/100-mm dish 24 h before transfection. For transfection of hOAT1-myc cDNA plasmid, a Lipofectamine 2000 reagent was used following manufacturer’s instructions. After 7–8 days of selection in medium containing 2 mg/ml Geneticin (G418; Invitrogen), resistant colonies were replated to 96 wells for cloning, expansion and analyzing positive clones.

Purification of Crude Membrane Proteins—LLC-PK1 cells or kidneys from Sprague-Dawley rats were homogenized in isolation buffer (250 mM sucrose, 10 mM triethanolamine with 1/100 protease inhibitor mixture) and centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was then centrifuged at 17,000 × g for 20 min at 4°C. The pellet was resuspended in appropriate volume of isolation buffer. Protein concentrations were determined using the Bio-Rad protein assay kit. The protein solutions were stored at −80 °C before further use.

Chemical Cross-linking—Membrane proteins were diluted to 1 mg/ml with cross-linking buffer (NaCl 150 mM, HEPES 100 mM, dithiothreitol 5 mM, EDTA 5 mM with 1/100 protease inhibitor mixture) and then incubated with 1–5 mM cross-linking reagents BS3, DSS, or DTSSP with end-over-end mixing for 30 min at room temperature. The cross-linking reaction was then stopped by incubation with 100 mM Tris/HCl, pH 7.5, for 15 min at room temperature. Appropriate volume of Laemmli buffer was added to denature the protein at 50 °C for 30 min, followed by electrophoresis and immunoblotting.

Chemical Cross-linking in 2% PFO—Membrane proteins were solubilized in lysis buffer containing 2% PFO, 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 10% glycerol with 1/100 protease inhibitor mixture for 30 min at room temperature. After clearance of insoluble materials by centrifugation at 11,000 × g for 20 min, supernatants were transferred to clean Eppendorf tubes and then incubated with 3 mM cross-linking reagent DSS with end-over-end mixing for 30 min at room temperature. The cross-linking reaction was then stopped by incubation with 100 mM Tris/HCl, pH 7.5, for 15 min at room temperature. Appropriate volume of Laemmli buffer was added to denature the protein at 50 °C for 30 min, followed by electrophoresis and immunoblotting.

Electrophoresis and Immunoblotting—Protein samples (with equal amount) were resolved on 7.5% SDS-PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween, washed, and incubated for 1 h at room temperature with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce, Inc.).

Co-immunoprecipitation—hOAT1-FLAG or rSat-1-FLAG was transfected into LLC-PK1 cells stably expressing hOAT1-myc. The cells were lysed in immunoprecipitation buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 0.5–1% Triton X-100 or 0.5% PFO, 2 mM EDTA, 10% glycerol, and 1/100 protease inhibitor mixture). Cell lysates were precleared with protein A-agarose beads to reduce nonspecific binding. The pre-cleared cell lysates were incubated with anti-myc antibody (1:100) for 1 h at room temperature. Appropriate volume of protein A-agarose beads were then added and mixed with end-over-end rotating at 4 °C for 1 h. Proteins bound to the protein A-agarose beads were eluted with Laemmli buffer containing β-mecapto-ethanol and analyzed by immunoblotting with horseradish peroxidase-conjugated anti-FLAG antibody (1:500, Sigma).

Gel Filtration Chromatography—Gel filtration chromatography was performed on an AKTA purifier system (Amersham Biosciences) with a Superdex 200 column. 100 μg of crude membrane proteins from LLC-PK1 cells stably expressing hOAT1-myc were solubilized in buffer containing 2% PFO, 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 10% glycerol with 1/100 protease inhibitor mixture for 30 min at room temperature. After clearance of insoluble materials by centrifugation at 11,000 × g for 20 min, the supernatant was injected into the column equilibrated with the running buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 5% glycerol, and 0.1% PFO). The elution was collected at 1 ml/fraction at the flow rate of 0.5 ml/min. Proteins were then precipitated with 10% trichloroacetic acid. The retention time of hOAT1 was determined by SDS-PAGE and immunoblotting of the collected fractions. Protein markers, including blue dextran (void volume marker), apoferritin, β-amylase, bovine serum albumin, ovalbumin, and carbonic anhydrase were separated under the same conditions and detected by the UV light detector (280 nm) of the AKTA purifier.

Cell Surface Biotinylation—Cell surface biotinylation was performed using the membrane-impermeable biotinylation reagents, sulfo-NHS-LC-biotin or biotin-LC-hydrazide. For labeling with sulfo-NHS-LC-biotin, LLC-PK1 cells stably expressing hOAT1-myc were placed on ice for two successive 20-min incubations with sulfo-NHS-LC-biotin (0.5 mg/ml in PBS/Ca²⁺/Mg²⁺, pH 8.0) with very gentle shaking. The reagent was freshly prepared in each incubation. After biotinylation, cells were briefly rinsed with 3 ml of PBS/Ca²⁺/Mg²⁺ containing 100 mM glycine and then incubated on ice with the same solution for 20 min to ensure complete quenching of the unreacted sulfo-NHS-LC-biotin. The cells were then dissolved on ice for 30 min in lysis buffer (0.5% Triton X-100, 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 10% glycerol with 1/100 protease inhibitor mixture). The lysed cells were removed by centrifugation at 11,000 × g for 20 min at 4 °C. At this stage, the supernatant was treated with (see below) or without cross-linking reagent. When not treated with cross-linker, the supernatant was directly incubated with anti-myc antibody (1:100) to immunoprecipitate hOAT1-myc and its associated proteins. The immunoprecipitates were analyzed by immunoblotting with horseradish peroxidase-conjugated streptavidin, and signals were detected by SuperSignal West Dura extended duration substrate kit. When the supernatant was treated with a thio-cleaveable cross-linker DTSSP, the cross-linked complexes were then incubated with anti-myc antibody (1:100) to immunoprecipitate hOAT1-myc and its associated proteins. The immunoprecipitates were then treated with 100 mM dithiothreitol at 37 °C for 1 h to break the disulfide bonds in cross-linker DTSSP, followed by electrophoresis and immunoblotting with horseradish peroxidase-conjugated streptavidin, and signals were detected by SuperSignal West Dura extended duration substrate kit.

For labeling with biotin-LC-hydrazide, LLC-PK1 cells stably expressing hOAT1-myc were incubated on ice with sodium periodate (10 mM in PBS/Ca²⁺/Mg²⁺, pH 7.0) for 30 min. The cells were washed three times with PBS and once with sodium acetate (pH 5.5). Then 1 ml of biotin-LC-hydrazide (2 mM in sodium acetate/Ca²⁺/Mg²⁺, pH 5.5) was added to each well and incubated on ice for 30 min. The unreacted biotin-LC-hydrazide was removed by washing the cells three times with PBS, pH 7.0. The cells were then dissolved on ice for 30 min in lysis buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10% glycerol with 1/100 protease inhibitor mixture). The unylsed cells were removed by centrifugation at 11,000 × g for 20 min at 4 °C. Supernatants were precleared with protein A-agarose beads and incubated with anti-myc antibody (1:100) to immunoprecipitate hOAT1-myc and its associated proteins. The immunoprecipitates were analyzed by immunoblotting with horseradish peroxidase-conjugated streptavidin, and signals were detected by SuperSignal West Dura extended duration substrate kit.
proteins was carried out by replacing the medium with fresh methionine-free Dulbecco’s modified Eagle’s medium supplemented with 100 μCi of [35S]methionine and incubated for 1 h at 37 °C and 5% CO2. The chase was started by aspirating the [35S]methionine-containing medium, followed by two PBS washes and incubation in the complete Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 12 h. The labeled cells were then washed twice with PBS and harvested for lysate preparation using the lysis buffer (10 mM NaCl, 10 mM Tris/HCl, pH 7.5, 2 mM EDTA, 0.5–1% Triton X-100, 10% glycerol with 1/100 protease inhibitor mixture). The unlysed cells were removed by centrifugation at 11,000 X g for 20 min at 4 °C. The supernatants were transferred to clean Eppendorf tubes and immunoprecipitated with anti-myc antibody (1:100) as described above. The immunoprecipitated proteins were denatured in Laemmli buffer for SDS-PAGE and autoradiography.

Transport Measurements—Uptake of [14C]PAH was initiated by adding uptake solution (PBS/Ca2+/Mg2+, pH 7.4, containing 5 mM glucose and 20 μM [14C]PAH) to either the basal or apical side of the monolayers. At times as indicated in the figure legends, the uptake was stopped by rapidly washing the cells 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. Uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (n = 3).

RESULTS

Generation of LLC-PK1 Cells Stably Expressing hOAT1-myc—One potential problem in studying the oligomerization of transporters is the use of a transient expression system, where much of the recombinant protein is not correctly processed, and therefore random aggregation of the proteins may occur. To circumvent this problem, we generated LLC-PK1 cells stably expressing hOAT1. To make such a cell line, we cloned the cDNAs encoding hOAT1 behind the cytomegalovirus promoter in the mammalian expression vector pcDNA3.1, which contains the neo gene for selection with G418. Myc tag was engineered into hOAT1 to facilitate the immunodetection of the hOAT1 protein, and we have previously showed that the myc-tagged hOAT1 retained the functional properties of its native (unmodified) form (16, 22). After 2 weeks of selection, clones exhibiting high levels of uptake of [14C]PAH, a prototypical organic anion, were chosen for further studies. Immunoblot analysis using crude membranes from hOAT1-myc-expressing cells revealed a myc antibody-reactive protein at ∼80 kDa (Fig. 1a, lane 1), which corresponded to the fully glycosylated form of the hOAT1 monomer (16). Such protein was not detected in pcDNA vector-transfected cells (Fig. 1a, lane 2). Therefore, hOAT1 in membranes of LLC-PK1 cells contained fully processed carbohydrates.

hOAT1 protein was further visualized by indirect immunolocalization using a fluorescence microscope (Fig. 1b). A top view of the hOAT1-myc-expressing cell monolayer showed a clear plasma membrane staining (bright fluorescence) with anti-myc antibody, whereas the pcDNA vector-transfected LLC-PK1 cells showed no detectable labeling.

Next, the functional localization of hOAT1 in polarized LLC-PK1 cells was examined. At confluence, LLC-PK1 cells form an epithelium with separate apical and basolateral membrane domains containing different complements of membrane proteins (23). The hOAT1 transporter in vivo is expressed exclusively at the basolateral membrane (1–3). The uptake of PAH, a prototypical organic anion, from either the basal or the apical side was measured in confluent monolayers of cells expressing hOAT1-myc or vector alone. Cells were grown on permeable membrane filter supports to provide independent access to either the apical or the basolateral membrane. As shown in Fig. 1c, a 10-min uptake of [14C]PAH from the basal side was significantly higher than that from apical side in cells expressing hOAT1-myc, whereas little uptake was observed in vector-alone-transfected cells. Therefore, hOAT1-myc was functionally expressed primarily on the basolateral membrane of polarized LLC-PK1 cells, which is consistent with its in vivo role as a basolateral organic anion transporter.

Chemical Cross-linking of Membrane Proteins—To explore whether hOAT1 forms oligomeric complexes, we used a strategy of chemical cross-linking, followed by electrophoresis and immunoblotting. As shown in Fig. 2a, cross-linking with BS3 in hOAT1-expressing LLC-PK1 cells converted the hOAT1 monomer (∼80 kDa) to proteins with molecular sizes corresponding to a trimer and a higher order of oligomer. Cross-linking using a different reagent, DSS, gave the same result (data not shown). This suggests that hOAT1 stably expressed in LLC-PK1 cells may form oligomeric complexes. A similar experiment was then performed with membrane proteins isolated from rat kidney. Upon cross-linking, the intensities of dimer and trimer increased at the expense of rat OAT1 monomer (∼80 kDa) (Fig. 2b), suggesting that OAT1, regardless of species origin, forms oligomers both in cultured cells and in kidney tissue. Such oligomerization, however, was not stabilized by disulfide bonds, because + reduction with β-mercaptoethanol had no effect on the oligomerization of hOAT1, which migrated with the same mobility in both cases. As a control, human IgG whose four subunits (two light chains of ∼25 kDa/each, and two heavy chains of ∼55 kDa/each) are linked by disulfide bonds dissociated into its subunits under the same condition (data not shown).

Gel Filtration Chromatography Analysis of hOAT1—The oligomeric status of hOAT1 was further analyzed by gel filtration chromatography using a Superdex 200 column after extraction with 2% PFO. PFO is a mild ionic detergent and does not absorb at 280 nm, the wavelength at which the UV light detector detected the proteins. When used at an appropriate concentration, PFO does not break the noncovalent interactions between protein subunits of an oligomer, and therefore has been
HOAT1 Forms Homooligomers

FIGURE 2. Oligomerization of HOAT1. a, cross-linking of membrane proteins from LLC-PK1 cells expressing HOAT1-myc. Total membrane proteins were cross-linked with a cross-linker BS3. The cross-linked proteins were then subjected to immunoblotting with anti-myc antibody. The apparent molecular masses of HOAT1 oligomers were estimated based on the linear regression of the molecular mass markers used. b, PFO extraction of HOAT1 from LLC-PK1 cell membranes. Crude membranes from HOAT1-expressing LLC-PK1 cells were treated with 2% PFO-containing buffer followed by centrifugation at 11,000 × g to separate the supernatant from the pellet. All supernatant and pellet were subjected to immunoblotting with anti-myc antibody. c, gel filtration chromatography of HOAT1. Top panel, the supernatant from 100 μg of membrane proteins extracted with 2% PFO was separated by gel filtration chromatography. The fractions were collected and precipitated with trichloroacetic acid for SDS-PAGE and immunoblot analysis. Bottom panel, retention times of markers blue dextran (81,000,000, as void volume marker), apoferritin (Ap, 443,000), β-amylose (Am, 200,000), bovine serum albumin (BSA, 66,000), ovalbumin (Ov, 45,000), carbonic anhydrase (Ca, 29,000). The apparent molecular masses of HOAT1 were estimated based on the linear regression of the relative retention time of the molecular mass markers. d, cross-linking of HOAT1 in detergent PFO. Membrane proteins from HOAT1-myc expressing cells were solubilized in 2% PFO, followed by cross-linking with 3 mM DSS. The proteins were then analyzed by electrophoresis and immunoblotting with anti-myc antibody.

successfully used to study the oligomeric status of many membrane proteins (20, 24, 25). We first tested the ability of PFO to extract HOAT1 from membranes of LLC-PK1 cells (Fig. 2c). PFO at concentration of 2% successfully extracted most of HOAT1, and the low speed centrifugation (11,000 × g) separated the extracted HOAT1 from the insoluble pellet. On the other hand, the majority of HOAT1 remained with the membrane pellet after low speed centrifugation if untreated by PFO. Therefore, we used 2% PFO to extract HOAT1 for further oligomeric analysis by gel filtration chromatography. As shown in Fig. 2d (top panel), most of HOAT1 was detected between fractions 11 and 12 ml, with a peak in the fraction of 12 ml. The peak fraction had an estimated molecular mass of 360 kDa. We did not detect any HOAT1 with the size of a monomer (~80 kDa), suggesting that the major form of HOAT1 in the plasma membrane is oligomeric.

The argument that HOAT1 detected in the peak fraction on the gel filtration column was oligomeric was further strengthened through a cross-linking study. In the same detergent (2% PFO) used for gel filtration chromatography, the cross-linking reagent was able to convert HOAT1 monomer (~80 kDa) to a putative dimer and trimer (Fig. 2c).

Association of HOAT1-FLAG with HOAT1-myc—The conclusion that HOAT1 forms multimeric complex was substantiated in an immunoprecipitation experiment using LLC-PK1 cells co-expressing FLAG-tagged HOAT1 and myc-tagged HOAT1. The FLAG-tagged HOAT1 cDNAs were transfected into LLC-PK1 cells stably expressing HOAT1-myc. Transfected cells were lysed with detergents Triton X-100 or PFO. HOAT1-myc was precipitated with anti-myc antibody (1:100). The immunoprecipitates were then separated by SDS-PAGE, followed by immunoblotting with horseradish peroxidase-conjugated anti-FLAG antibody (1:500). As shown in Fig. 3a, in cells co-expressing HOAT1-myc and HOAT1-FLAG, HOAT1-FLAG readily co-immunoprecipitated with HOAT1-myc, whereas no FLAG band was labeled in cells expressing HOAT1-FLAG alone, demonstrating specific association between HOAT1-myc and HOAT1-FLAG. Because the experiments were carried out in either Triton X-100 or PFO, and the FLAG tag was added to either the amino terminus or the carboxyl terminus of HOAT1, these studies suggest that the type of the detergent used and the positions of the FLAG tag at HOAT1 did not affect the nature of HOAT1 oligomerization. We also transfected FLAG-tagged rat sulfate-anion antiporter rSat-1-FLAG into cells stably expressing HOAT1-myc. rSat-1 was previously shown to be expressed at the basolateral membrane of LLC-PK1 cells (26), the same membrane where HOAT1 is expressed. As shown in Fig. 3b, although rSat-1 was clearly expressed at the cell surface as determined by cell surface biotinylation, it was not detected in the immunoprecipitate. Therefore, rSat-1-FLAG did not associate with HOAT1-myc under such condition. This observation strengthened our conclusion on the specific association between HOAT1-FLAG and HOAT1-myc. If HOAT1 is a homooligomer, immunoprecipitation of one subunit must lead to a co-immunoprecipitation of the other. Therefore, the co-immunoprecipitation experiment indicated that HOAT1 might form a homooligomer.

Cell Surface Biotinylation and Metabolic Labeling of LLC-PK1 Cells Stably Expressing HOAT1—The possibility that HOAT1 forms a homooligomer was further examined by two different approaches. In the first approach, we analyzed the membrane bound fractions of HOAT1. We
HOAT1 Forms Homooligomers

labeled cell surface proteins with two membrane-impermeable biotinylation reagents sulfo-NHS-LC-biotin or carbohydrate-specific biotin hydrazide. Sulfo-NHS-LC-biotin selectively labels the lysine residue, whereas biotin hydrazide specifically labels carbohydrates. HOAT1 is a glycoprotein containing lysine residues and therefore can be labeled by both reagents. We reasoned that any HOAT1-associated membrane protein, as long as it contains either carbohydrates or a lysine residue, should be biotinylated by these reagents, should be co-immunoprecipitated with HOAT1, and consequently should be detected on immunoblot by horseradish peroxidase-conjugated streptavidin.

The immunoprecipitates were treated with reducing reagent dithiothreitol to cleave the disulfide bond present in the cross-linker, followed by immunoblotting with horseradish peroxidase-conjugated streptavidin. The immunoprecipitates were treated with reducing reagent dithiothreitol to cleave the disulfide bond present in the cross-linker, followed by immunoblotting with horseradish peroxidase-conjugated streptavidin. Under such conditions, we did not detect other proteins except HOAT1 in the cross-linked complex (Fig. 4c).

DISCUSSION

Abundant evidence exists to indicate that many transporters function as oligomers (19–21). However the information on the structural assembly of any cloned OATs is completely unknown up to date. Therefore, the major aim of this study was to investigate whether HOAT1 forms an oligomeric complex in the cell membrane. For that purpose, combined approaches of chemical cross-linking, gel filtration chromatography, co-immunoprecipitation, cell surface biotinylation, and metabolic labeling were employed. One potential problem encountered in studying the oligomerization of transporters is the use of a transient expression system, where much of the recombinant proteins is not correctly processed, and therefore random aggregation of the proteins may occur. To circumvent this problem, we established LLC-PK1 cells stably expressing HOAT1. LLC-PK1, a pig proximal tubule cell line, offers several useful advantages for study of the cloned organic anion transporter. (a) They have many characteristics of renal proximal tubules and have been very useful in understanding other renal epithelial transport processes and cellular functions, including organic cation transport.
HOAT1 Forms Homooligomers

(27). (b) This cell line does not express endogenous OATs (28). Therefore, expression of hOAT1 in LLC-PK1 cells will allow us to dissect the transport characteristics of hOAT1 in a relevant mammalian system without the possibly confounding effects of other organic anion transporters. Functional analysis showed that substantial hOAT1-dependent PAH uptake was detected at the basolateral surface, in agreement with its physiological role as a basolateral organic anion transporter. Therefore, hOAT1-expressing LLC-PK1 cells are a suitable model system for the characterization of hOAT1 oligomerization.

The cross-linking studies presented in Fig. 2a with membrane proteins from hOAT1-expressing LLC-PK1 cells demonstrated oligomerization of hOAT1 in these cells. The result was further confirmed with naturally occurring OAT1 from rat kidney (Fig. 2b), providing the physiological relevance for our study. The oligomers are not disulfide-bonded, because removing the reducing reagent β-mercaptoethanol did not promote oligomerization (data not shown). However, the non-covalent interactions between the subunits were not strong enough to withstand the effects of SDS, which is probably the reason that only hOAT1 monomers were observed on a SDS-PAGE without cross-linking.

As one of the complementary approaches to cross-linking, gel filtration chromatography was used to analyze the oligomeric status of hOAT1 (Fig. 2). In this experiment, PFO, a mild ionic detergent, was used to extract hOAT1 from cell membranes. PFO has been successfully used to study the oligomeric status of many membrane proteins (20, 24, 25). The major peak fraction of hOAT1 has an estimated molecular mass ~360 kDa, which is larger than hOAT1 trimer observed in cross-linking studies. This may result from the association of solubilized membrane proteins with detergent and possibly lipid. The argument that hOAT1 detected in the peak fraction on the gel filtration column was oligomeric was further strengthened through cross-linking of hOAT1 monomer to putative dimer and trimer in the same detergent mass.

The oligomerization hypothesis was reinforced by the ability to co-immunoprecipitate two differentially tagged hOAT1 (Fig. 3). Co-immunoprecipitation of hOAT1-FLAG with hOAT1-myc was indeed due to a specific interaction, because (a) in the absence of hOAT1-myc, precipitation of hOAT1-FLAG by anti-myc antibody did not occur, and (b) the FLAG-tagged rsat1 did not co-immunoprecipitate with hOAT1-myc under the same experimental condition. These experiments were carried out in the presence of either a mild ionic detergent, PFO, or a nonionic detergent, Triton X-100. Both of these detergents seem to fully preserve the subunit interaction of hOAT1, suggesting that the formation of hOAT1 oligomers is not because of the use of various detergents. hOAT1-FLAG also co-immunoprecipitated with hOAT1-myc in another cell system, a monkey kidney cell line, Cos-7 (data not shown), indicating that the association between hOAT1 subunits is not cell-specific.

The homooligomeric nature of hOAT1 was demonstrated by two different approaches: cell surface biotinylation with membrane impermeable reagents and metabolic labeling with 35S-labeled methionine. These two approaches allow separate analyses of the functional membrane-bound fractions of hOAT1 and its total cellular pools. Both approaches resulted in the isolation of a single labeled polypeptide species with a molecular mass of 80 kDa (Fig. 4), the size of the hOAT1 monomer, favoring the notion that the hOAT1 oligomer consists of only hOAT1 subunits. We are currently investigating whether each subunit of hOAT1 contains a single pore itself and allows the translocation of organic anions, a mechanism mimicking water channel CHIP28 (29), or whether several subunits are required to form the pore, as in K+ channels (30).

It is important to emphasize that the current study was carried out in kidney LLC-PK1 cells. The cell culture-based system is an essential model system for the basic characterization of cloned transporters in isolation and may reflect the in vivo situation where OAT exists alone, such as OAT2 in the liver and OAT4 in the placenta. However, such a system might not completely reflect the native membrane environment of the tissue of interest in terms of (i) how highly the individual transport protein is expressed, and (ii) any as yet unknown cell-type-specific protein-protein interactions, especially when several OATs co-exist at the same membrane of the same cell type, such as OAT1 and OAT3 in the kidney and brain. Under such conditions, whether different OAT isoforms associate with one another to form heterooligomers is an important question we are trying to address. In conclusion, this is the first study demonstrating that hOAT1 exists in the plasma membrane of kidney LLC-PK1 cells as a homooligomer, possibly trimer, and higher order of oligomer.

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