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The flounder organic anion transporter fOat has sequence, function, and substrate specificity similarity to both mammalian Oat1 and Oat3

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Abstract

The flounder renal organic anion transporter (fOat) has substantial sequence homology to mammalian basolateral organic anion transporter orthologs (OAT1/Oat1 and OAT3/Oat3), suggesting that fOat may have functional properties of both mammalian forms. We therefore compared uptake of various substrates by rat Oat1 and Oat3 and human OAT1 and OAT3 with the fOat clone expressed in *Xenopus* oocytes. These data confirm that estrone sulfate is an excellent substrate for mammalian OAT3/Oat3 transporters but not for OAT1/Oat1 transporters. In contrast, 2,4-dichlorophenoxyacetic acid and adefovir are better transported by mammalian OAT1/Oat1 than by the OAT3/Oat3 clones. All three substrates were well transported by fOat-expressing *Xenopus* oocytes. fOat K_m values were comparable to those obtained for mammalian OAT/Oat1/3 clones. We also characterized the ability of these substrates to inhibit uptake of the fluorescent substrate fluorescein in intact teleost proximal tubules isolated from the winter flounder (*Pseudopleuronectes americanus*) and killifish (*Fundulus heteroclitus*). The rank order of the IC_{50} values for inhibition of cellular fluorescein accumulation was similar to that for the K_m values obtained in fOat-expressing oocytes, suggesting that fOat may be the primary teleost renal basolateral Oat. Assessment of the zebrafish (*Danio rerio*) genome indicated the presence of a single Oat (zfOat) with similarity to both mammalian OAT1/Oat1 and OAT3/Oat3. The puffer fish (*Takifugu rubripes*) also has an Oat (pfOat) similar to mammalian OAT1/Oat1 and OAT3/Oat3 members. Furthermore, phylogenetic analyses argue that the teleost Oat1/3-like genes diverged from a common ancestral gene in advance of the divergence of the mammalian OAT1/Oat1, OAT3/Oat3, and, possibly, Oat6 genes.

Keywords

OAT1; OAT3; renal; isolated tubules

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The vertebrate kidney has a potent secretory capacity speeding the elimination of anionic xenobiotics and metabolites (see reviews, Refs. 6,15,30,36,40). The basolateral uptake of organic anions from the blood into the tubular epithelial cell is coupled to the in > out dicarboxylate gradient via organic anion/dicarboxylate exchange. Two organic anion transporters (Oat), namely, OAT1/Oat1 (*SLC22A6*) and OAT3/Oat3 (*SLC22A8*), are principally responsible for basolateral organic anion uptake by the proximal tubule (2,37)

OAT1/Oat1 and OAT3/Oat3 transporters that mediate the basolateral step have been cloned in several mammals, including rat (17,33,41), human (8,9,14,19,29), mouse (3,18), rabbit (1, 20), and monkey (42). In addition, clones of Oat1 have been obtained from pig (13) and opossum (partial sequence; accession no. AJ308236). In 1997, soon after the first Oat was cloned from rat (rOat1), a renal organic anion transporter was cloned from the winter flounder (fOat; Ref. 45). When expressed in *Xenopus* oocytes, fOat transports *p*-aminohippurate (PAH), generating a positive inwardly directed current (5). Like mammalian OAT1/Oat1 and OAT3/Oat3, fOat is a dicarboxylate exchanger (7). However, upon the cloning of mammalian OAT3/Oat3, it became apparent that fOat has substantial sequence homology with both mammalian OAT1/Oat1 and OAT3/Oat3 clones (on average, 64.4 and 61.8%, respectively; Table 1).

The purpose of the present study was to compare the functional characteristics of fOat with its mammalian orthologs OAT1/Oat1 and OAT3/Oat3. We measured uptake of various substrates by mammalian OAT1/3 orthologs and the fOat clone, all expressed in *Xenopus* oocytes. Specificities of cloned transporters were then compared with transport by intact teleost kidney tubules [isolated from winter flounder (*Pseudo-pluronectes americanus*) and killifish (*Fundulus heteroclitus*)] by following the ability of these agents to inhibit tubular uptake of the fluorescent fOat substrate fluorescein. To obtain additional insight into the number of teleost Oat transporters, we searched the recently published zebrafish (*Danio rerio*) genome for Oat1/3-like genes and identified a single locus on chromosome 21. With genomic and phylogenetic analyses, the zebrafish Oat1/3-like protein product was compared with mammalian OAT/Oat transporters, as well as fOat and an Oat1/3-like transporter present in the genome of another teleost, the puffer fish (*Takifugu rubripes*). Together, these data support expression of a single Oat1/3 ortholog in teleost fish and indicate that this ortholog has a broad substrate specificity that allows it to transport substrates characteristic of both mammalian transporters, OAT1/Oat1 and OAT3/Oat3.

MATERIALS AND METHODS

[³H]estrone sulfate (50 Ci/mmol) and *p*-[³H]aminohippurate (20 – 40 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). 2,4-[³H]dichlorophenoxyacetic acid (2,4-D; 20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Both ³H-labeled (9.1–12.3 Ci/mmol) and unlabeled 9-(2-phosphonylmethoxyethyl)-adenine (adefovir) were purchased from Moravak Biochemicals (Brea, CA) or donated by Gilead Pharmaceuticals (Foster City, CA). Collagenase A was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Fluorescein was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade or better.

Oocyte Isolation and Injection

Female *Xenopus laevis* were purchased from either Xenopus One (Dexter, MI) or NASCO (Fort Atkinson, WI). Animals were tricaine (0.3%) anesthetized, decapitated, and pithed, and the ovaries were removed. All animal procedures were carried out in accordance with protocols approved by the National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee. Oocytes were defolliculated by collagenase A digestion followed by incubation in K₂HPO₄ buffer, as described previously (9,29,38). For recovery, defolliculated oocytes were stored at 18°C overnight in oocyte Ringer 2 (OR-2; in mM: 82.5 NaCl, 2.5 KCl,

1 Na₂HPO₄, 3 NaOH, 1 CaCl₂, 1 MgCl₂, 1 Na-pyruvate, and 5 HEPES, pH 7.6) supplemented with 5% horse serum and 50 µg/ml gentamycin. Stage V and VI oocytes were then microinjected with either HPLC water or 20–25 ng of capped cRNA.

RNA Synthesis

For all OAT1/3 clones, capped cRNA was synthesized from linearized cDNA with the mMessage mMachine in vitro transcription kit (T7 kit for all clones; Ambion, Austin, TX). The human OAT1 (hOAT1) cDNA (accession no. AF124373) was a gift from Tomas Cilhar (Gilead Sciences, Foster City, CA) and was isolated as described previously (1,3). The fOat (Z97028), rOat1 (AF008211), and rOat3 (AB17466) cDNAs were produced in our laboratories, as described previously (2,4,45).

The hOAT3 clone (AB042505) was recently isolated in our laboratory using RT-PCR. Briefly, a human kidney cDNA template was generated by reverse transcription (Powerscript RT system; Clontech, Palo Alto, CA) of human kidney total RNA (Clontech) according to the manufacturer's instructions. Oligonucleotide primers specific to the 5' and 3' untranslated regions of the hOAT3 sequence [5'-CTG AGC TGC CCT ACT ACA G-3' (bases 1–19) and 5'-GCT GTT TAT TGA AAC CTC CC-3' (bases 2134–2153)] were synthesized (Qiagen, Germantown, MD) and used in the Expand long template PCR system (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The resulting PCR product was purified (QIAEX II agarose gel extraction kit; Qiagen) and subcloned into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Resulting cDNAs were sequenced to confirm fidelity to the published sequence and orientation in vector by automated sequencing (BigDye terminator cycle sequencing ready reaction kit; ABI Biosystems, Forest City, CA) at the NIEHS core facility (Research Triangle Park, NC). A single nucleotide error (G to A, at position 704) was corrected by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) according to the manufacturer's protocols, using the oligonucleotide primers 5'-CAT TAC CCT GAG CAC CGT CAT CTT G-3' and 5'-CAA GAT GAC GGT GCT CAG GGT AAT G-3'. The resulting cDNA was confirmed as 100% identical to the published sequence.

Oocyte Transport

Radiolabeled substrates—Transport assays were conducted 3–4 days after injection. Oocytes were separated into groups of 10 in 24-well plates, and each well was washed three times with 2 ml of unsupplemented OR-2, followed by 1 ml of OR-2 containing the indicated substrate/isotope. After 20 min of incubation at room temperature, oocytes were rinsed five times with 2 ml of ice-cold 0.1 M MgCl₂. Each oocyte was then transferred to a 7-ml scintillation vial and lysed in 200 µl of 10% aqueous SDS. After complete lysis, 4 ml of EcoLume scintillation fluid (ICN Biomedical, Cleveland, OH) were added to each vial, and samples were counted for radioactivity with a Tri-Carb 2900TR liquid scintillation analyzer (Packard, Meriden, CT).

Fluorescent substrate—Five to six days postinjection, fOat-injected oocytes were incubated in 5 µM fluorescein or 5 µM fluorescein and 200 mM probenecid for at least 1.5 h at room temperature in OR-2. Fluorescein accumulation in fOat-injected oocytes was imaged using a Zeiss 510 laser scanning confocal microscope (× 10 objective, 488-nm excitation; Carl Zeiss, Jena, Germany). Because yolk platelets preclude imaging deep into the oocytes, optical slices were taken 10 µm from the surface of the oocytes, i.e., 10 µm above the supporting coverslip. As a control, noninjected oocytes were treated with 5 µM fluorescein and imaged under the same conditions.

Winter Flounder and Killifish Proximal Tubule Fluorescein Assays

Cellular and luminal accumulation of fluorescein in teleost proximal tubules were assayed as described previously (23,25). Briefly, killifish and flounder were obtained from the Mount Desert Island Biological Laboratory (MDIBL; Salisbury Cove, ME). The flounder were housed on site in seawater aquaria at MDIBL, whereas the killifish were transferred to NIEHS and housed in aquaria containing artificial seawater until use. Fish were tricaine (0.3%) anesthetized and decapitated, and kidneys were removed and bathed in Tris-Forster's saline (in mM: 140 NaCl, 2.5 KCl, 1.5 CaCl₂, 1 MgCl₂, and 20 Tris, pH 8.25). Proximal tubules were manually teased from renal tissue with fine forceps under a dissection microscope to remove adherent connective and hematopoietic tissue. In addition, the flounder tubules, which have a higher density of hematopoietic tissue, were rapidly triturated (10×) through a p5000 pipette tip (Rainin, Woburn, MA). After dissection, tubules were transferred to a Teflon chamber with a glass coverslip bottom. Each of these chambers was prefilled with 1 ml of Tris-Forster's saline with fluorescein (1 μM for killifish, 2 μM for flounder) and estrone sulfate, cimetidine, or 2,4-D as indicated. Tubules were incubated in the dark for 45 min (killifish) or 1 h (flounder). The tubules were then viewed through a ×25 water-immersion objective (0.8 NA) on a Zeiss inverted laser scanning confocal microscope (model LSM 510 or LSM 5 PASCAL; Jena, Germany). Samples were illuminated with a 488-nm argon line set to low intensity (< 2% maximum) passed through a 488-nm dichroic beam splitter. The signal was then passed through a 505-nm long-pass filter to a photomultiplier tube. The gain on the photomultiplier tube was adjusted so that the mean luminal fluorescence did not exceed 200 (fluorescence unit scale of 0 to 255), and images were collected as an average of 4 frames and saved in 8-bit format. Fluorescence image intensities were measured with Zeiss LSM 510 software. For each tubule, areas (> 30 μm²) from the cellular and luminal regions were selected and mean fluorescence was recorded. The data are presented as the cumulative mean (±SE) of each region.

Statistical Analysis

Results are presented as representative data from at least two experiments. Kinetic constants were calculated from fOat-mediated uptake with Kaleidagraph 3.0 (Synergy Software, Reading, PA) using iterative curve fitting. Statistical analysis was performed with Graph-Pad Prism (San Diego, CA) or Microsoft Excel (Redmond, WA). Differences among means were considered statistically significant at $P < 0.05$.

Bioinformatics

Slc22 members were identified from zebrafish by BLASTP analysis, using the lineage-specific motifs STIVTEWD/NLVC and ELYPTVIR (11,32) as queries and using the PAM30 matrix with gap costs set at 9 for existence and 1 for extension. Accessions were required to contain both motifs (with a minimum 7/11 similar amino acids and 6/8 similar amino acids, respectively). Accessions that met these criteria were used as queries in a TBLASTN search of the *D. rerio* reference genome [zebrafish build 1 genome database (reference assembly only), posted July 1, 2005; <http://www.ncbi.nlm.nih.gov/projects/genome/guide/zebrafish>]. The resulting contigs were analyzed for Oat protein-coding regions. One of the putative zebrafish Oat transporters, BC095733, is an experimentally derived cDNA (accession no. NW_634899). The associated protein (accession no. AAH95733) includes the erroneously translated polyA tail. For this work, AAH95733 was truncated at the polyA tail.

The accession numbers fOat (CAB09724), zfOat (NP_996960), and pfOat (SINFRUP00000071613) were aligned in pairwise comparisons with each other and with representatives of OAT/Oat1–6 and URAT1/Urat1 by using ClustalW (Vector NTI Advance 10.0.1; Invitrogen) and the BLOSUM62mt2 matrix under the default conditions. BLASTP analysis of the nonredundant protein database with mOat6 (NP_941052) as the query allowed

us to identify three more Oat6 members. Rat Oat6 (XP_219524), dog Oat6 (XP_854865), and cow Oat6 (XP_604969) were included in the Oat6 comparisons (Table 1).

Multiple sequence alignments were performed using ClustalW (Vector NTI Advance 10.0.1) under the default conditions [including the Neighbor Joining method of Saitou and Nei (31) and Kimura's correction (16)]. The resulting guide tree was exported to Treeview 1.6.6 to create a radial dendrogram (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

RESULTS

As shown in Fig. 1, the pattern of transport for the three substrates tested differed between the mammalian OAT1/Oat1 and OAT3/Oat3 (human and rat) clones expressed in *Xenopus* oocytes. Rat and human OAT1/Oat1 transporters showed little or no transport of estrone sulfate (1 μ M). Conversely, the OAT3/Oat3 transporters mediated markedly higher uptake of estrone sulfate than water-injected oocytes (Fig. 1), indicating that its basolateral transport is a reflection of OAT3/Oat3 activity. The nucleoside phosphonate adefovir also seemed to discriminate between the two orthologs. Adefovir (30 μ M) was well transported by both OAT1/Oat1 transporters. However, adefovir transport by OAT3/Oat3 transporters was more limited. Finally, transport of the herbicide 2,4-D (10 μ M) was similar to that of adefovir.

Transport of these compounds was then assessed in fOat-expressing *Xenopus* oocytes (Fig. 1). Estrone sulfate (1 μ M), the mammalian OAT3/Oat3 substrate, was robustly transported by fOat-expressing oocytes, with uptake 125-fold greater than in water-injected controls. Likewise, adefovir (30 μ M), the OAT1/Oat1 substrate, was avidly transported by fOat, with uptake 98-fold greater than in water-injected controls. Transport of 50 μ M 2,4-D was also significantly (5.5-fold) greater in fOat-expressing oocytes than in water-injected controls.

Kinetic constants were determined for fOat transport of estrone sulfate and adefovir. The fOat-mediated transport of estrone sulfate had an apparent K_m value of $9.6 \pm 1.7 \mu$ M and a V_{max} value of $1.6 \pm 0.1 \text{ pmol} \cdot \text{oocyte}^{-1} \cdot \text{min}^{-1}$ (Fig. 2). For adefovir, K_m and V_{max} values were $112 \pm 37 \mu$ M and $4.7 \pm 0.5 \text{ pmol} \cdot \text{oocyte}^{-1} \cdot \text{min}^{-1}$, respectively. We attempted to measure kinetic constants for 2,4-D, but high background in water-injected controls precluded this determination. Therefore, we measured 2,4-D inhibition of 10 μ M PAH uptake. The IC_{50} value for 2,4-D was $5.7 \pm 0.1 \mu$ M (data not shown).

Teleost renal tubules transport fluorescein effectively, and fluorescein transport is inhibited by PAH and lithium and induced when glutarate is added to the bathing medium, indicating transport by Oat proteins (24). Moreover, fluorescein is an established substrate for both mammalian OAT1/Oat1 and OAT3/Oat3 (22,35). Thus, provided fluorescein is a substrate for fOat, it should be possible to use inhibition of fluorescein transport by intact renal tubules to assess the impact of Oat transporters on basolateral organic anion transport in the intact teleost tubule (39). To confirm that fluorescein is a substrate for fOat, we assessed fluorescein uptake by fOat-expressing *Xenopus* oocytes. As shown in Fig. 3, oocytes expressing fOat readily took up fluorescein, as shown by fluorescence microscopy. Furthermore, fluorescein uptake was not seen in noninjected oocytes and was completely blocked by 200 μ M probenecid.

When fluorescein uptake was measured in tubules isolated from winter flounder, 100 μ M estrone sulfate inhibited both cellular and luminal fluorescein accumulation by ~75% (Fig. 4). Fluorescein uptake was completely abolished by 500 μ M estrone sulfate (data not shown). We also tested cimetidine, which has previously been identified as a fOat substrate (4). Cimetidine is not an organic anion but, rather, a weak organic base that is well transported by mammalian OAT3/Oat3 (12) and acts as an inhibitor of uptake by hOAT3 but not hOAT1 (34). Both cellular (51%) and luminal (38%) fluorescein accumulation were inhibited by 300 μ M cimetidine, and uptake was virtually abolished by 3 mM cimetidine (Fig. 4).

Fluorescein uptake was also examined in killifish renal proximal tubule. This model has been used extensively because of its superior viability and ease of preparation (23,25). Moreover, the Oat1 substrate adefovir is known to inhibit fluorescein transport in intact killifish tubules (23). We therefore compared tubular transport by tissue from this teleost to that of the winter flounder. Estrone sulfate (Fig. 5A) inhibited both cellular and luminal accumulation with IC_{50} values of 3.7 and 2.1 μ M, respectively. For cimetidine, inhibition of cellular and luminal fluorescein accumulation was essentially identical (Fig. 5B), with an IC_{50} value of 325 μ M for both compartments. Inhibition of luminal accumulation by 2,4-D occurred at lower concentrations (IC_{50} 4.3 μ M) than inhibition of cellular accumulation (IC_{50} 8.9 μ M).

Whole genome analysis is not yet possible for the winter flounder; however, a reference teleost genome is available for the zebrafish, *D. rerio*. The zebrafish genome was searched for all Slc22 family members, yielding considerable information regarding the organic anion and cation transporters in this model organism. Loci encoding Oat, organic cation transporters (Oct), and organic cation/carnitine transporters (Octn) were identified in the genome. All results were initially characterized by sequence similarity, and although representatives of other transporter families were identified, only two accessions had extensive similarity to Oat1 or Oat3. These were chosen for further investigation.

NP_996960 was encoded on contig NW_634139 on chromosome 21 and appeared to be an Oat1/3-like protein (zfOat). The predicted length of the protein was 560 amino acids. This protein had both lineage-specific motifs (10/11 and 8/8 identical, respectively). A second contig, NW_634899, encoded a protein (accession no. AAH95733) that in phylogenetic analysis fell between the Oat6 node and the fish Oat1/3-like node (zfOatX; see Fig. 7). zfOatX is 51% identical to zfOat protein. zfOatX may, among other possibilities, represent the fish version of Oat6, a diverged fish Oat1/3-like protein or an Oat protein that evolved only in fish and for which no mammalian ortholog exists. Final determination is not yet possible.

A second complete fish genome, puffer fish (*T. rubripes*) was also available (<http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html>). A search of the puffer fish predicted proteins revealed an Oat1/3-like protein (SINFRUP00000071613; pfOat). Flounder, zebrafish, and puffer fish Oat were aligned using ClustalW (Vector NTI; Invitrogen) in pairwise comparisons with each other and mammalian OAT/Oat proteins. In pairwise alignments, fOat (CAB09724), zfOat, and pfOat had identity scores ranging from 66.2 to 71.4% (Table 1). Fish Oat1/3-like proteins had identity scores ranging from 43.2 to 46.4 when compared with OAT3/Oat3 proteins and scores from 46.3 to 48.7 with OAT1/Oat1 proteins. Interestingly, the percent identity of fish Oat1/3-like proteins with the newly reported Oat6 (26) was also very high (45.1–46.9%).

The fish Oat1/3-like proteins contain previously identified motifs common to Slc22 family members. A multiple sequence alignment of portions of the fish Oat1/3-like members with mammalian OAT1/Oat1 and OAT3/Oat3 proteins is shown in Fig. 6. The MFS (major facilitator superfamily motif, $GX_3GX_4GX_3DRFGRR$; Ref. 21) and the ASF (amphiphilic solute facilitator) lineage-specific motifs ELYPTVIR (11,32) and STIVTEWD/NLVC (11, 32) are shown. fOat, zfOat, and pfOat retained all three motifs. Although sequence motifs to differentiate OAT family members from each other are not yet reported (11), all OAT3/Oat3 members had aspartic acid in the first position of the ASF motif, whereas other OAT/Oat proteins had serine. Interestingly, only fish Oat1/3-like proteins had isoleucine in the fourth position of the same motif. We also noted that only OAT1/Oat1 proteins had methionine (7th position), alanine (13th position), and leucine (16th position) in the MFS motif. The alanine and leucine were shared between OAT1/Oat1 and Oat6. These amino acid differences may not have functional significance. However, as additional sequences become available, these may begin to define motifs to distinguish OAT/Oat members from each other.

A phylogenetic tree was developed that included the fish Oat1/3-like proteins and representatives of the mammalian OAT/Oat1–6 and URAT1/Urat1. In phylogenetic analysis, there is clear separation of the fish Oat transporters from the mammalian OAT1/Oat1 or OAT3/Oat3 groups and from the mammalian Oat6 group (see Fig. 7). The results are consistent with an OAT/Oat1/3 gene duplication that occurred after the divergence of fish and terrestrial vertebrates. Conversely, zOat2 sorts with its OAT2/Oat2 orthologs (Fig. 7), as expected since this member was likely to originate before the fish/terrestrial vertebrate radiation.

DISCUSSION

As shown previously (34,42) and in Fig. 1, estrone sulfate is a specific mammalian OAT3/Oat3 substrate, i.e., well transported by mammalian OAT3/Oat3 transporters with little or no OAT1/Oat1-mediated transport. Adefovir appears to be well transported by mammalian OAT1/Oat1 transporters but not by the OAT3/Oat3 transporters. The anionic herbicide 2,4-D is handled by both isoforms, with preferential handling by OAT1/Oat1 (Fig. 1; Ref. 42). PAH, at 10 μM , is handled by both isoforms but shows more robust transport by the mammalian OAT1/Oat1 transporters (Refs. 34,37; data not shown). In contrast to its mammalian orthologs, fOat has the capacity to transport all of these substrates well (Fig. 1; Refs. 4,45). In particular, it transports adefovir (mammalian OAT1/Oat1 substrate) and estrone sulfate (mammalian OAT3/Oat3 substrate) very effectively. Not only does fOat transport substrates for both mammalian orthologs, it has a similar affinity for these substrates as well. For example, for the OAT3/Oat3-specific substrate estrone sulfate, reported K_m values from *Xenopus* oocyte expression data range from 2.3 to 33 μM (includes hOAT3 and rOat3 values) (8,12,17). The K_m value for fOat was in the middle of this range at 9.6 μM . For adefovir, the mammalian OAT1/Oat1 marker, reported K_m values from *Xenopus* oocyte expression data range more broadly (30 μM for hOAT1 to 270 μM for rOat1) (9), but once again the fOat K_m value of 112 μM is well within that range. For the mammalian OAT/Oat1/3 substrate PAH, OAT1/Oat1 K_m values from *Xenopus* oocyte expression data are generally in the range of 4–16 μM in human and pig (1,13,14,19,44) with a broader range of values reported for rat (11–70 μM) (29,33,41,46). For OAT3 orthologs, PAH K_m values from *Xenopus* oocyte expression data are somewhat higher, ranging from 65 (rat) to 87.2 μM (human) (8,17). Once again, the fOat K_m value for PAH is in the same range, 20–58 μM (7,45). Flounder Oat also shares other common OAT/Oat1/3 substrates, including glutarate, furosemide, ochratoxin A (reviewed in Ref. 5a), α -ketoglutarate (42,45), fluorescein (Fig. 3; Refs. 22,35), and 2,4-D (Fig. 1; Refs. 28,42,43).

These results indicate that the teleosts may have a single renal basolateral Oat performing the functions of the mammalian OAT/Oat1/3 pair and suggest that the two basolateral mammalian orthologs may be the descendants of a more ancient gene similar to that for fOat. Certainly, the functional evidence presented (Figs. 1–5) argues that fOat is the only basolateral Oat1/3 in this species. In fact, the rank orders for the kinetic constants determined for fOat expressed in oocytes and the IC_{50} values measured in the intact teleost tubules are remarkably similar. For example, the high affinity of estrone sulfate for expressed fOat is reflected in its low K_m value (9.6 μM) and its low IC_{50} value against proximal tubule fluorescein accumulation (3.7 μM). Conversely, the fOat apparent K_m value for adefovir was 112 μM in oocytes, almost identical to its IC_{50} value of 100 μM in the intact killifish proximal tubule (23), reflecting the lower affinity of the transporter for this substrate. Thus it appears that the basolateral organic anion transport observed in the teleost can be wholly described by the activity of a single Oat.

Fitting nicely with the above assessment is the recent genomic analysis of Eraly et al. (10). They showed that OAT1/Oat1 and OAT3/Oat3 are not only highly homologous but that they occur as tightly linked paralogs on human chromosome 11 and mouse chromosome 19, where they are separated by only small intergenic distances (8.3 kb for humans and 7.5 kb for mice). Furthermore, comparative genomic analysis of the 5' flanking regions upstream of OAT1/Oat1

and OAT3/Oat3 in humans and mice suggests the paralogs may share common regulatory elements and that they may be the result of a gene duplication of an ancestral gene. It also was demonstrated recently that these mammalian carriers share an identical transport mechanism and function as organic anion/dicarboxylate exchangers (36), the same mechanism as fOat (2,6). Certainly, these findings are consistent with the functional data presented in this study and argue that the teleost Oat (exemplified by fOat) may illustrate the properties of such an ancestral carrier. The broader substrate specificity of the fOat provides an interesting contrast to the molecular specialization seen in mammalian orthologs. Similarly, only a single Oat1/3-like locus was identified in zebrafish. Sequence analysis of fOat with the zfOat1/3-like protein and the *T. rubripes* (Japanese puffer fish) Oat1/3-like protein indicates that zebrafish, puffer fish, and flounder Oat transporters are closely related. Phylogenetic analysis supports the hypothesis that the common ancestor of fish and terrestrial vertebrates had a single gene with sequence and possible substrate specificity similar to Oat1, Oat3, and perhaps Oat6 (Fig. 7).

In conclusion, this study provides evidence that fOat transports substrates for mammalian OAT1/Oat1 and OAT3/Oat3 with similar affinities to these orthologs. Sequence comparisons of teleost Oat proteins with mammalian OAT1/Oat1 and OAT3/Oat3 indicate the presence of a single Oat protein with homology to both members. Thus both functional and genomic evidence argue that the basolateral teleost Oat orthologs share properties of the ancestral gene that gave rise to the mammalian OAT1 and OAT3 orthologs.

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Fig. 1.

Uptake of estrone sulfate (ES), adefovir, and 2,4-dichlorophenoxyacetic acid (2,4-D) by *Xenopus* oocytes expressing mammalian OAT1/Oat1, OAT3/Oat3, and flounder renal organic anion transporter (fOat) cDNAs. Concentrations tested were 1 μ M ES, 30 μ M adefovir and 10 μ M 2,4-D; h, human; r, rat. fOat 2,4-D data were generated at 50 μ M, and its uptake has been divided by 5 to allow expression on the same scale as mammalian OAT/Oat data. Fold stimulation over the water controls is shown above each bar. Bars reflect the means $\pm$ SE of 5–10 replicates from a representative experiment repeated in oocytes from 3 animals. Data are presented as mediated uptake (i.e., $\text{pmol} \cdot \text{oocyte}^{-1} \cdot 20 \text{ min}^{-1}$ after subtraction of mean uptake by water-injected control oocytes).

Fig. 2.

Kinetics of ES and adefovir uptake by fOat. A 20-min time period was used. Mediated uptake (□) was calculated by subtracting the mean uptake in water-injected oocytes (▲) from the mean uptake in fOat-injected oocytes (●). Points reflect the means $\pm$ SE of 7–10 replicates from a representative experiment repeated in oocytes from 4 animals. Responses in fOat-expressing oocytes were compared with those in water-injected (control) oocytes using unpaired nonparametric (Mann-Whitney) t -test (* $P < 0.05$).

Fig. 3.

Fluorescein accumulation by fOat-expressing oocytes. *A*: noninjected oocytes exposed to 5 μ M fluorescein showed no accumulation after at least 1.5 h of incubation. FI, fluorescein. *B*: uptake of 5 μ M fluorescein in fOat-injected oocytes as demonstrated by fluorescein fluorescence. *C*: incubation of fOat-injected oocytes with 200 μ M probenecid, an organic anion transporter inhibitor, prevented the uptake of 5 μ M fluorescein. Images are representative of 4 oocytes (bar, 200 μ m). Pro, probenecid.

Fig. 4.

Inhibition of cellular and luminal fluorescein (2 μ M) accumulation in isolated winter flounder proximal tubules by ES and cimetidine. Fluorescence intensities were normalized to the untreated control cells. Bars reflect the pooled mean $\pm$ SE of 5–17 tubule replicates observed in 2–3 fish. Responses were normalized to the fluorescence intensity in cells of control tubules (100%). Responses were analyzed using unpaired nonparametric (Mann-Whitney) *t*-test, comparing the test concentrations to control (no inhibitor) for cell and lumen conditions (**P* < 0.05).

Fig. 5.

Inhibition of cellular (●) and luminal (□) fluorescein (1 μM) accumulation in isolated killifish proximal tubules by ES (A), cimetidine (B), and 2,4-D (C). Points reflect the pooled mean (includes 2 animals) $\pm$ SE of 6–12 tubule replicates from a representative experiment repeated 2–3 times. Graphs at *left* were analyzed using unpaired nonparametric (Mann-Whitney) *t*-test, comparing the test concentrations to control (no inhibitor) for cell and lumen conditions (**P* < 0.05). Graphs at *right* were analyzed using paired nonparametric (Wilcoxon) *t*-test, comparing responses at each concentration for the cellular and luminal components (**P* < 0.05). RFU, relative fluorescence units.

	ASF motif STIVTEWD/NLVC	MFS motif GXXXGXXXGXXXDRFGRR	ASF motif ELYPTVIR
pOAT1	STIVTEWDLVC	GVLLGAMIFGYLANRLGRR	EVYPTMIR
hOAT1	STIVTEWDLVC	GVLLGAMVFGYLADRLGRR	ELYPTMIR
rbOAT1	STIVTEWDLVC	GVLIGAMVFGYLADRLGRR	ELYPTMIR
mOAT1	STIVTEWNLVC	GVLLGAMMFGYLADRLGRR	ELYPTMIR
rOAT1	STIVTEWNLVC	GVLLGAMVFGYLADRLGRR	ELYPTVIR
fOat	ST I SEWHLVC	GVLVGALLFGGLSDRYGRR	EL F PTIIR
pfOat	SS I SDWDLVC	GVLVGAGIFGALSDRYGRR	ELYPTVIR
zfOat	ST I TEWDLVC	GVLVGAIVFGGLSDRFGR	ELYPTVIR
rbOAT3	DTIVTEWDLVC	GILIGGLVLGDLSDRFGRK	ELFPTVIR
hOAT3	DSIVTEWDLVC	GILIGGLVLGDLSDRFGR	ELYPTVIR
pOAT3	DSIVTEWDLVC	GILVGGLVLGALSDFGRK	ELYPTVIR
mOAT3	DTIVTEWDLVC	GILVGGPVFGELSDRFGRK	ELYPTVLR
rOAT3	DTIVIEWDLVC	GILVGGPVIGELSDRFGRK	ELYPTVLR

Fig. 6.

Amino acid sequence comparison of winter flounder (fOat), zebrafish (zfOat), and puffer fish (pfOat) with mammalian OAT1 and OAT3 representatives. Only the lineage-specific motifs are shown. Amino acids that differ from the motifs are in bold type.

Fig. 7.

Phylogenetic analysis depicting divergence among teleost and mammalian organic anion transporters. Representatives of teleost Oat1/3-like proteins, mammalian OAT members 1– 6, and URAT1 were aligned with their teleost counterparts. Teleost Oat names are in bold type, whereas teleost Oat1/3-like proteins are designated in bold italic type. NCBI accession numbers are as follows: CAB09724 (fOat), SINFRUP00000071613 (pfOat), NP_996960 (zfOat), AAH95733 (zfOatX), NP_004781 (hOAT1), CAB62587 (rbOat1), NP_032792 (mOat1), NP_058920 (rOat1), NP_001001261 (pOat1), NP_445989 (rOat2), NP_659105 (mOat2), AAZ79452 (rbOat2), NP_006663 (hOAT2), XP_696026 (zfOat2), CAD34035 (rbOat3), NP_004245 (hOAT3), NP_999620 (pOat3), NP_112471 (mOat3), NP_112622 (rOat3), NP_060954 (hOAT4), NP_543142 (hOAT5), NP_659034 (mOat5), BAB78471 (rOat5), XP_854865 (dOat6), NP_941052 (mOat6), XP_219524 (rOat6), BAB96750 (hURAT1), and NP_033229 (mUrat1) (f, flounder; pf, puffer fish; zf, zebrafish; h, human, rb, rabbit; m, mouse; r, rat; p, pig; d, dog).

Table 1
Sequence homology of teleost and mammalian organic anion transporters

	fOat		zfOat		pfOat	
	%Similar	%Identical	%Similar	%Identical	%Similar	%Identical
fOat						
zfOat	77.7	66.3				
pfOat	82.2	71.4	77.9	66.2		
OAT1/Oat1 (6)	64.4	46.3	67.1	48.7	63.6	46.6
OAT2/Oat2 (5)	52.4	35.4	53.8	37.2	53.7	35.8
OAT3/Oat3 (5)	61.8	45.6	61.5	46.4	60.3	43.2
OAT4/Oat4 (1)	56.3	39.7	56.8	39.1	57.5	34.3
hOAT5 (1)	51.1	35.2	51.9	34.5	52.2	34.3
m/rOat5 (2)	51.9	34.1	53.7	34.3	53	32.8
Oat6 (4)	60.9	45.1	63.0	46.9	61.8	46.9

Similarity and identity between flounder Oat (fOat) and their mammalian orthologs. fOat amino acid sequences were aligned with each other and with mammalian representatives of the known OAT/Oat transporters to determine the percent similarity and identity between them. Where more than one representative of a family was used, results were averaged. The number of sequences used in the comparison is shown in parentheses following the family name (*n*). Oat, organic anion transporter; f, flounder; zf, zebrafish; pf, puffer fish; h, human (OAT); m, mouse; r, rat.