Molecular Characterization and Tissue Distribution of a New Organic Anion Transporter Subtype (oatp3) That Transports Thyroid Hormones and Taurocholate and Comparison with oatp2*

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Two complementary DNAs for the organic anion transporter subtypes oatp2 and oatp3, which transport thyroid hormones as well as taurocholate, were isolated from a rat retina cDNA library. The sequence of oatp2 is identical to that recently reported (Noe´, B., Hagenbuch, B., Stieger, B., and Meier, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10346–10350), whereas the sequence of oatp3 is novel. oatp3 consists of 670 amino acid residues and exhibits a structural architecture common to the organic anion transporter family, possessing the 12 putative membrane-spanning segments. Oocytes injected with oatp2 and oatp3 cRNAs showed taurocholate uptake in a saturable manner. The oatp2 and oatp3 cRNA-injected oocytes also showed significant uptake of both thyroxine and triiodothyronine. Northern blot and in situ analyses showed that the oatp2 mRNA was widely expressed in neuronal cells of the central nervous system, especially in the hippocampus, cerebellum, and choroid plexus as well as in the retina and liver. The oatp3 mRNA was highly expressed in the kidney and moderately abundant in the retina. This suggests that oatp2 and oatp3 are multifunctional transporters involved in the transport of thyroid hormones in the brain, retina, liver, and kidney.

A homeostatic system controls the fluid environment in the brain and keeps its chemical composition relatively constant compared with that of plasma. One mechanism is the blood-brain barrier, which selectively transports chemical substances via capillary endothelial cells (1). A second essential component is the choroid plexus (blood-cerebrospinal fluid barrier), which secretes or takes up specific chemical substances (2). Although the presence of specific transporting mechanisms has long been postulated, little is known about their molecular identity. Recent molecular biological studies revealed the organic anion transporter family: the Na¯-independent organic anion-transporting polypeptide oatp1 from rat liver, which transports bile acid, bromosulfophthalein (BSP), and conjugated and unconjugated steroid hormones (3, 4); the kidney-specific transporter OAT-K1, which transports methylxenate in the basolateral membrane of renal tubules (5); and the prostaglandin transporter (6). Moreover, physiological studies have suggested the presence of other members of the organic anion transporter family (7). Noe et al. (8) have recently reported that a new organic anion transporter subtype (oatp2) is present in rat brain and liver and that the oatp2-expressed oocytes transported cardiac glycoside as well as taurocholate. However, the endogenous substrate of oatp2 and the regional distribution in the brain have not been revealed.

It has been suggested that thyroid hormones are transported into the brain via the blood-brain barrier (9) or via the choroid plexus (10). To reveal this mechanism, we focused on the retina. In the retina, the retinal pigment epithelium is the unique source of transthyretin synthesis, and it serves to transport thyroxine (T4) across the blood-retina barrier (11). According to this functional homology between the choroid plexus epithelium and the retinal pigment epithelium, we performed polymerase chain reaction (PCR)-based screening for an organic anion transporter subtype in the retina.

In this paper, we have isolated and characterized oatp2 and a new organic anion transporter family subtype (termed oatp3) from rat retina, and this is the first report identifying the molecules involved in thyroid hormone transport.

**Experimental Procedures

AmpTM DNA amplification reagent kit according to the following conditions: 1.5–2 µg of genomic DNA was used in 50 µl reaction mixture. The reaction mixture contained 150–200 ng of the DNA template, 5 pmol of each primer, 1 unit of Taq polymerase, 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 3 mM MgCl₂, 0.05% Triton X-100, and 1 µg/ml bovine serum albumin at 95 °C for 3 min to activate Taq polymerase, followed by 35 cycles, each of 94 °C for 30 sec., 54 °C for 30 sec., and 72 °C for 1 min. An aliquot of the PCR products was electrophoresed on an agarose gel. The amplified DNA (~1500 base pairs (bp)) was excised from the gel and subcloned into pBlueScriptII KS(+) by a single-strand ligation method. By sequencing, one clone (psOATP2), corre-

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This nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U95011 and AF041105.

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1 The abbreviations used are: BSP, bromosulfophthalein; T₄, thyroxine; T₃, 3,5,3’-triiodo-L-thyronine; PCR, polymerase chain reaction; bp, base pair(s).
sponding to amino acid residues 62–545 of oatp2 (8), was found to show 78.4% identity to rat oatp1 at the amino acid level.

cDNA Isolation—

A rat retina cDNA library was constructed from fractions containing 2-kilobase pair cDNAs using a ZAPII vector (Stratagene) (12), and 83105 independent clones were screened with the 1.5-kilobase pair cDNA fragment of psOATP2. Hybridization was carried out in 25% formamide solution at 42 °C as described (13), and filter washing was performed in a solution containing 2×SSC and 0.1% SDS at 55 °C. By a series of screenings, 12 hybridization-positive clones were isolated. Eight of the 12 clones were identified to have the same insert as psOATP2, and the remaining clones were found to show a novel sequence. In each group, clones pR8 and pR1, both of which contained the largest insert, were chosen for further analysis. The sequences of both clones were determined with an ABI Prism™ 377 DNA sequencer (Perkin-Elmer).

Functional Characterization of oatp2 and oatp3 in Xenopus Oocytes—

cRNA was synthesized in vitro using T7 RNA polymerase in the presence of the cap analogue m7GpppG from linearized pR8 or pR1. Transcribed cRNA (25 ng) was injected into defolliculated Xenopus laevis oocytes. Injected oocytes were cultured for 2–3 days in Barth’s medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, and 15 mM Hepes, pH 7.6). The uptake (10 min) of [3H]taurocholate, [125I]T4, or 3,5,3'-[125I]triiodo-L-thyronine (T3) (all from NEN Life Science Products) was assayed at room temperature in 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 m M Hepes, and 5 mM Tris-HCl, pH 7.5. The oocytes injected with water were used as a control. Each value represents the mean ± S.E. of at least three experiments in different oocytes. The statistical significance was tested by unpaired t-test.

Northern Blot Analysis—

2 m g of poly(A)+ RNAs from tissues were analyzed on a 1% agarose gel and blotted onto a nitrocellulose membrane. The membranes were hybridized with the 32P-labeled psOATP2 cDNA fragment under mild conditions and washed to a lower stringency. The blots were exposed to x-ray film for detection of the bands.

**Fig. 1. Amino acid comparison of oatp2 and oatp3.** The two sequences are aligned by inserting gaps (-) to achieve the maximum homology. Exact matches and conservative substitutions are shown by bars and colons, respectively. The 12 putative transmembrane segments (I–XII) were assigned on the basis of hydrophobicity analysis and the sequence comparison of the other organic anion transporters; the termini of these segments were tentatively defined. The putative transmembrane regions are indicated by solid lines. Triangles, potential N-glycosylation sites; asterisks, possible phosphorylation sites.
Two Organic Anion Transporters Transport Thyroid Hormones

Electrophoresed on 1.0% agarose gel and transferred to a nylon membrane. Hybridization was performed with a 32P-labeled fragment from the ~900-bp HincII-NcoI fragment of the 3’-noncoding region for pR2 or the ~900-bp Smal-Smal fragment of the 3’-noncoding region for pR1 in a hybridization buffer containing 50% formamide, 5x SSC, 5x Denhardt’s solution, and 1% SDS at 42 °C overnight. The hybridized filter was washed in 0.2 x SSC and 1% SDS at 65 °C and exposed to a film at ~80 °C for 3 h or overnight. Both probes have <4% identity to each other or to any member of the organic anion transporter family to avoid any cross-hybridization.

In Situ Hybridization of oatp2—For in situ hybridization of the oatp2 mRNA, the same 3’-noncoding HincII-NcoI fragment of pR8 was subcloned, and a radiolabeled antisense cRNA was synthesized by T7 RNA polymerase using γ-32P-CTP (NEN Life Science Products). The resulting RNAs were hydrolyzed to make ~150-nucleotide fragments. The brain sections of male Sprague-Dawley rat were fixed and incubated with radiolabeled probe in the hybridization solution (50% formamide, 2 x SSC, 10 mM Tris-HCl, 1 x Denhardt’s solution, 10% dextran sulfate, and 0.2% SDS) at 55 °C for 6 h, washed subsequently with 2 x SSC containing 10 mM β-mercaptoethanol at 50 °C, treated with RNase A, and washed with 0.1 x SSC at 60 °C for 2 h. The sections were dehydrated, exposed to Hyperfilm™-β-max (Amersham Pharmacia Biotech) for 14 days, dipped into Kodak NTB-2 (Eastman Kodak Co.) diluted 1:1 with distilled water, developed after a 6-week exposure, and counterstained with hematoxylin-eosin.

RESULTS

cDNA Isolation and Structure of oatp2 and oatp3—A rat retina cDNA library was screened by hybridization with the fragment derived from PCR subclone psOAPT2 under low stringency condition. Twelve clones were isolated and rescued into pBluescript SK(−). Eight of the 12 clones showed restriction patterns corresponding to oatp2 (8), and one representative clone (pR8) was used for further analysis. pR8 was determined to be composed of ~3.8 kilobase pairs, and the amino acid sequence of pR8 was identical to that of oatp2 (661 amino acids with M₉ = 73,199.09) (8), except for several nucleotide differences in the 5’-noncoding region. The restriction patterns of the remaining four clones were novel, and the length of each clone was almost identical. Among the four clones, one clone (pR1), which has the largest insert, showed a nucleotide sequence that was similar but not identical to any of the organic anion transporter family cDNAs, including oatp2. The deduced amino acid sequence of pR1 consists of 670 amino acids (M₉ = 74, 643.86), encoding novel organic anion transporter subtype oatp3. Fig. 1 shows the deduced amino acid sequence alignment of oatp2 and oatp3. oatp2 and oatp3 showed an identity of 82% at the amino acid level. oatp3 also shows 80% amino acid identity to oatp1 (3), 76.7% to rat kidney OAT-K1 (5), and 33.8% to the rat prostaglandin transporter (6). Hydrophobicity analyses of oatp3 predicts 12 hydrophobic segments, characteristic of the organic anion transporter family. For oatp3, four N-glycosylation sites are predicted, and there are three potential phosphorylation sites for cAMP-dependent protein kinase and six potential phosphorylation sites for protein kinase C in the cytosolic hydrophilic loops (14, 15).

Pharmacological Characterization of oatp2 and oatp3—Based on the structural similarity among oatp1, oatp2, and oatp3, we first examined the uptake of [3H]taurocholate in the oatp2 or oatp3 cRNA-injected oocytes. The oatp2 cRNA-injected oocytes increased taurocholate uptake 18-fold above that of the water-injected oocytes: 0.11 ± 0.007 versus 0.006 ± 0.001 pmol/oocyte/min at 30 μM [3H]taurocholate (p < 0.01). Taurocholate transport followed saturation kinetics with an apparent Km of 35.2 ± 8.9 μM (Fig. 2a). To characterize the pharmacological properties of oatp2-mediated taurocholate uptake, we next examined the effects of other compounds (200 μM) on [3H]taurocholate (1 μM) uptake. Taurocholate, cholate, BSP, and 17β-estradiol glucuronide significantly inhibited [3H]taurocholate uptake (data not shown), whereas p-aminobenzoic acid did not. These results are comparable to those obtained with oatp1 (16) and to those reported by Noe et al. (8). In addition, T₄ and T₃ also significantly inhibited oatp2-mediated [3H]taurocholate incorporation (data not shown).

The oatp3 cRNA-injected oocytes also transported [3H]taurocholate with an apparent Km of 17.9 ± 3.32 μM, which was half of that for oatp2-mediated uptake (Fig. 2b). This oatp3-mediated taurocholate uptake was inhibited by BSP, but not by p-aminobenzoic acid. We also tested whether oatp3 transports other compounds. No oatp3-mediated uptake was found for radiolabeled tryptophan, phenylalanine, tyrosine, and indomethacin.

Do oatp2 and oatp3 transport thyroid hormones? To answer this question, we next studied the uptake of thyroid hormones in the oatp2- or oatp3-expressing oocytes. The oatp2 cRNA-injected oocytes transported [125I]T₄ and [125I]T₃ significantly more than the water-injected oocytes (Fig. 3, a and b). These transports of thyroid hormones were saturable with increasing substrate concentrations. The apparent Km values for [125I]T₄ and [125I]T₃ in oatp2 cRNA-injected oocytes were 6.53 ± 2.56 and 5.87 ± 1.06 μM, respectively. Furthermore, the uptake of [125I]T₄ by oatp2-expressing oocytes was inhibited by unlabeled T₄ and T₃ (data not shown). We next investigated whether oatp2-mediated thyroid hormone uptake is dependent on ex-
tracellular Na⁺. When extracellular Na⁺ (100 mM) was substituted with isosmotic choline⁺, the uptake of [125I]T₄ was only slightly inhibited (15.2 ± 0.1%), and this difference was insignificant (p > 0.1). Similarly, substitution of extracellular Cl⁻ with gluconate (16.1 ± 0.06%) did not insignificantly affect T₄ uptake (p > 0.1). Thus, T₄ transport by oatp2 is independent of Na⁺ or Cl⁻ in the external medium.

The oatp3 cRNA-injected oocytes also transported [125I]T₄ and [125I]T₃ (Fig. 3, c and d), with an apparent Kₘ of 4.93 ± 1.79 μM for [125I]T₄ and 7.33 ± 2.34 μM for [125I]T₃. These values were comparable to those for oatp2, and the oatp3-mediated T₄ and T₃ uptake was also independent on external Na⁺ or Cl⁻ in the external medium.

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**Distribution of oatp3 mRNAs—**Relative levels of the oatp2 and oatp3 mRNAs were analyzed by Northern blot hybridization (Fig. 4, a and b). A single ~3.9-kilonucleotide oatp2 mRNA band was found in the brain, retina, and liver (Fig. 4a). No significant hybridization signals were observed in the heart, lung, and kidney. On the other hand, the analysis of oatp3 mRNA yielded two hybridization bands with estimated mRNA sizes of ~2.8 and ~3.9 kilonucleotides in the retina and kidney (Fig. 4b). The oatp3 mRNA expression level was less in the retina. The two different size oatp3 mRNAs are probably derived from the same gene because both bands were seen under high stringency filter washing conditions using the 3’-noncoding region that has <48% identity to any other organic anion transporter family member. A faint single band was also detected in the liver (~3.9 kilonucleotides). No obvious band was seen in other tissues. Identical oatp2 and oatp3 mRNA tissue distributions were also obtained using the rat Multiple tissue Northern™ blots filter purchased from CLONTECH (data not shown).

Although Noe et al. (8) showed the expression of oatp2 mRNA in the brain, the regional expression was not determined. To examine this, in situ hybridization was performed using a specific riboprobe prepared from the noncoding region of the oatp2 cDNA. As shown in Fig. 5a, the oatp2 mRNA was widely distributed throughout adult rat brain. Specifically, intense signals were observed in the hippocampal pyramidal and dentate granule cells (Fig. 5, a and c) and cerebellar Purkinje and granule cell layers (Fig. 5, a and d). Moderate expression signals were also observed in the olfactory neuronal layers, anterior olfactory nuclei, striatum, cerebral cortex, thalamic and hypothalamic nuclei, and various brain stem nuclei (Fig. 5a). In addition to the neuronal expression, the oatp2 mRNA was moderately expressed in the choroid plexus (Fig. 5a, indi-
icated by the arrow). An almost identical pattern of oatp2 mRNA expression was observed using another riboprobe (774-bp HindIII-HincII fragment of oatp2) (data not shown). All hybridization signals were completely abolished when hybridization was performed in the presence of a 200-fold excess of unlabeled probes (Fig. 5b), confirming the specificity of the localization pattern of the oatp2 mRNA.

**DISCUSSION**

This paper presents the characterization and tissue distribution of two cDNA clones encoding rat organic anion transporter subtypes oatp2 and oatp3 isolated from rat retina. The putative amino acid sequence of oatp3 was novel, whereas that of oatp2 was identical as recently reported (8). oatp3 is unique compared with other members of the organic anion transporter family in its tissue distribution.

Both oatp2 and oatp3 appear to be responsible for transporting organic anions and thyroid hormones for the following reasons. First, the expression experiments with oatp2 and oatp3 using *Xenopus* oocytes showed that both oatp2 and oatp3 transport[^3H]taurocholate. oatp2 transports taurocholate with a $K_m$ value similar to that reported (35.2 ± 8.9 versus 34 μM) (8). The oatp3 cRNA-injected oocytes also transported[^3H]taurocholate, but the apparent $K_m$ value was half of that for oatp1- and oatp2-mediated uptake (8). Second, a series of cis-inhibitory studies on the oatp2-expressing oocytes showed that the oatp2-mediated uptake of[^3H]taurocholate was markedly inhibited by the addition of taurocholate, cholate, BSP, or 17β-estradiol glucuronide as well as T₄ and T₃. The oatp3-mediated uptake of[^3H]taurocholate was markedly inhibited by the addition of taurocholate, cholate, BSP, or 17β-estradiol glucuronide as well as T₄ and T₃. The oatp3-mediated taurocholate uptake was also inhibited by BSP. Third, the oatp2- and oatp3-expressing oocytes facilitated the uptake of thyroid hormones (T₄ and T₃) with simple Michaelis-Menten kinetics, and the apparent $K_m$ values are comparable to each other. Fourth, Northern blot analysis showed that the oatp2 mRNA is distributed in the brain, retina, and liver. The oatp3 mRNA is exclusively expressed in the retina and kidney. On the other hand, the oatp1 mRNA is expressed in the liver, kidney, brain, skeletal muscle, and colon (3). Finally, *in situ* hybridization analysis indicated that the oatp2 mRNA is widely distributed in neuronal cells of many brain regions as well as in the choroid plexus.

This is the first report identifying the molecules responsive for transporting thyroid hormones across cell membrane. The transport of thyroid hormones across the plasma membrane determines the intracellular concentration of these hormones and hence the activation of the nuclear T₃ receptor. The existence of mechanisms regulating the transport of thyroid hormones has been suggested in cerebrocortical neurons (17), as well as in the choroid plexus.

The $K_m$ values for T₄ and T₃ of oatp2 and oatp3 were similar to those for neural cells (19) and hepatocytes (21), but 10-fold higher than those reported by Chantoux et al. (17) and Blondeau et al. (20). We do not know what accounts for these differences. In our study, the oatp2-mediated uptake of T₄ was not dependent on extracellular Na⁺, whereas the transporting mechanisms of thyroid hormones are heterogeneous in Na⁺ dependence: Na⁺ dependent (21), Na⁺ independent (19, 20, 24) and mixed (18, 23). In addition, it has been reported that oocytes injected with rat liver poly(A)⁺ RNA showed Na⁺ dependent uptake of T₄ and T₃ (25); however, the functional fraction size is different from that of the oatp2 or oatp3 mRNA. Therefore, the molecules responsible for transporting thyroid hormones are suggested to be heterogeneous.

The uptake of thyroid hormones in the native tissues appears to be inhibited by a variety of structurally unrelated drugs, including non-bile acid cholephils such as BSP, nonsteroidal anti-inflammatory drugs (20, 24), diphenylhydantoin (26), and propranolol (27). In our study, the oatp2-mediated uptake of taurocholate was inhibited by some of these compounds, but no oatp3-mediated uptake was found for tryptophan, phenylalanine, tyrosine, and indomethacin. In the oatp1-expressing mammalian cells, T₄ did not exhibit a cis-inhibitory effect for BSP uptake (28). Thus, further investigations will be required to understand the role and functions of the other members of the organic anion transporter family.

The oatp2 mRNA is distributed exclusively in the brain, retina, and liver. In the central nervous system, most of the nuclear T₃ is derived from local T₄ deiodination (29). However, most of the intracellular T₃ in the liver and muscle is derived from plasma (30). Accordingly, the abundant expression of the oatp2 mRNA in the brain and liver implies that this molecule should regulate the availability of thyroid hormone in these tissues. Thyroid hormones also play an essential role in neural function of the mammalian central nervous system, particularly during a critical period of its development (31, 32). The absence of thyroid hormone causes serious damages to structural development and organization of the brain (especially the hippocampus and cerebellum), including biochemical maturation, and leads to irreversible mental retardation (33).

In the hippocampus, the oatp2 mRNA is predominantly expressed in the pyramidal cells of CA1–4 and in the granule cells of the dentate gyrus. In the cerebellum, the oatp2 mRNA is highly expressed in Purkinje cells. The prominent expression of the oatp2 mRNA in these thyroid hormone-sensitive neurons further suggests that oatp2 may play a critical role in neuronal development and maintaining cell function.

The oatp2 mRNA was also moderately distributed in the choroid plexus. This is consistent with the notion that thyroid hormones are transported into the brain via the blood-brain barrier (9) or via the choroid plexus (10). *In situ* hybridization of rat oatp1 revealed that rat oatp1 mRNA is also expressed in the choroid plexus (34). Although the probe for rat oatp1 (positions 2031–2090) is 80–95% identical among organic anion transporter family members, the probe only detected signals in
used the 3' non-coding region of oatp2 that has <48% identity to oatp1, OAT-K1, and oatp3. On the other hand, Noé et al. (8) used nucleotides 1–360, which has >80% identity to oatp1 (82%) and OAT-K1 (87%). In their report, the size of the major band in the kidney was smaller than that in the brain. It is possible that their probe cross-hybridized to the other member of the organic anion transporter family, e.g. oatp3 or OAT-K1. In addition, since the probes for oatp3 clearly hybridized to the kidney mRNA, the mRNAs used were not degenerate in our study. The same result was obtained using the rat multiple-tissue Northern filter. In both cases, mRNA qualities were confirmed by β-actin (data not shown). Based on our results, we suggest that the oatp2 mRNA is not expressed in the kidney as much as shown for oatp2 (8).

The oatp3 mRNA is exclusively distributed in the kidney. Most of the T4 secreted from the thyroid is deiodinated in peripheral tissues. The liver and kidney are the major peripheral organs producing T3 from T4 (35). Thus, the specific expression of oatp3 mRNA in the kidney suggests an essential role for oatp3 in transporting thyroid hormones from the circulation to the deiodination sites in the kidney.

Individuals with Refetoff’s syndrome, characterized by resistance to thyroid hormone, exhibit reduced clinical and biochemical activities of thyroid hormone action relative to the circulating hormone level (36). Several pathophysiological mechanisms have been suggested to account for the thyroid hormone resistance seen in Refetoff’s syndrome patients. One possible mechanism is reduced hormone availability to tissues due to impaired thyroid hormone entry into cells (36). Accordingly, functional and genetic investigations of oatp2 and oatp3 may also help us to understand the etiology of such disorders and to aid in clinical diagnosis.

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