

Immunohistochemical distribution and functional characterization of an organic anion transporting polypeptide 2 (oatp2)

Masayuki Kakyo^{a,b}, Hiroyuki Sakagami^c, Toshiyuki Nishio^{a,d}, Daisuke Nakai^e,
Rie Nakagomi^e, Taro Tokui^e, Takeshi Naitoh^b, Seiki Matsuno^b, Takaaki Abe^{a,*},
Hiromu Yawo^a

^aDepartment of Neurophysiology, Tohoku University School of Medicine, 2-1 Seiryō-cho, Aoba-ku, Sendai 980-8575, Japan

^b1st Department of Surgery, Tohoku University School of Medicine, Sendai 980-8575, Japan

^cDepartment of Histology, Tohoku University School of Medicine, Sendai 980-8575, Japan

^dDepartment of Pediatrics, Tohoku University School of Medicine, Sendai 980-8575, Japan

^eAnalytical and Metabolic Research Laboratories, Sankyo Co., Ltd., Tokyo 140-8710, Japan

Received 19 November 1998; received in revised form 25 January 1999

Abstract The rabbit polyclonal antibody against rat organic anion transporting polypeptide 2 (oatp2) was raised and immunoaffinity-purified. Western blot analysis for oatp2 detected two bands (~74 and 76 kDa) in rat brain and a single band (76 kDa) in the liver. By immunohistochemical analysis, the oatp2 immunoreactivity was specifically high at the basolateral membrane of rat hepatocytes. Functionally, the oatp2-expressing oocytes were found to transport dehydroepiandrosterone sulfate, δ_1 opioid receptor agonist [D-Pen²,D-Pen⁵]enkephalin, Leu-enkephalin, and biotin significantly, as well as the substrates previously reported. These data reveal the exact distribution of the rat oatp2 at the protein level in the liver, and that oatp2 appears to be involved in the multispecificity of the uptaking substrates in the liver and brain.

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Key words: Organic anion transporting polypeptide 2; Basolateral membrane; Rat liver; Thyroid hormone; Steroid hormone; Dehydroepiandrosterone sulfate; [D-Pen²,D-Pen⁵]Enkephalin

1. Introduction

Recently, we and another group have independently isolated a cDNA for Na⁺-independent organic anion transporting polypeptide 2 (oatp2) from rat retina [1] and brain [2]. Oocytes injected with the oatp2 cRNA showed taurocholate, thyroxine (T₄), triiodothyronine (T₃) and conjugated steroid (estradiol 17 β -glucuronide and estrone sulfate) uptake in a saturable manner [1,2]. Northern blot analysis and in situ hybridization revealed that the oatp2 mRNA was highly expressed in the brain and liver [1], whereas Noé et al. detected an additional band in the kidney [2]. Although this discrepancy may be attributed to the specificity of the probes, no study has been made on the distribution of oatp2 at the protein level.

In this report, to identify the expression of oatp2 at the protein level, Western blot and immunohistochemical analyses

of oatp2 were performed. The oatp2 immunoreactivity was high in the liver, particularly at the basolateral membrane of the hepatocytes. In contrast, no oatp2 immunoreactivity was detected in the kidney, confirming our previous report on the oatp2 mRNA distribution. The detailed pharmacological characterization of oatp2 was also examined in *Xenopus laevis* oocytes in order to identify the additional substrates for oatp2.

2. Materials and methods

2.1. Preparation of rabbit antibodies for rat oatp2

A peptide containing 12 amino acids (CTEVLRSKVTED, position 650–661) at the carboxy-terminus of rat oatp2, a region that is different from both rat oatp1 and oatp3, was synthesized [1]. This peptide was linked to the maleimide-activated keyhole limpet hemocyanin (KHL; Pierce). The KHL-linked peptide (1 mg/injection) was emulsified by mixing with an equal volume of the Freund's adjuvant and injected into female rabbits. Boosts were performed at 2, 6, and 8 weeks, and animals were killed at 10 weeks. The antibodies were affinity-purified using CNBr-activated Sepharose CL-4B (Amersham Pharmacia Biotech) coupled with synthetic peptides according to the standard procedure [3].

2.2. Preparation of liver membranes

Male Sprague-Dawley rats (200 g) were anesthetized and the liver was resected. The liver was homogenized in a buffer containing 0.23 M sucrose, 5 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A. The homogenate was centrifuged at 3000 \times g for 15 min and the supernatant was further centrifuged at 100 000 \times g for 30 min. The resultant pellet is referred to as the crude membrane fraction and was resuspended in a buffer of 0.23 M sucrose, 5 mM Tris-HCl, pH 7.5, and 2 mM EDTA [4].

2.3. Western blot analysis

Western blot analysis was performed by a modification of a previously reported method [3]. Briefly, 10 μ g of crude membranes were solubilized by a sample buffer (2% SDS, 125 mM Tris-HCl, pH 7.4, 20% glycerol, 2% 2-mercaptoethanol) at room temperature for 5 min and applied onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The blots were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS)-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.5, containing 0.1% Tween 20) overnight at 4°C and incubated with the affinity-purified anti-oatp2 antibody (1 μ g/ml) for 1 h at room temperature. The blots were then incubated with anti-rabbit IgG conjugated with horseradish peroxidase (HRP, 1:5000 dilution, Amersham Pharmacia Biotech) for 1 h at room temperature. Enhanced chemiluminescence kit was used for detection (Amersham Pharmacia Biotech). To identify the antibody specificity, the anti-oatp2 antibody was incubated with 10 μ g of the immunogen peptide before use.

*Corresponding author. Fax: (81) (22) 717-8154.

E-mail: takaabe@mail.cc.tohoku.ac.jp

Abbreviations: oatp, organic anion transporting polypeptide; DHEAS, dehydroepiandrosterone sulfate; DPDPE, 2-D-penicillamine, 5-D-penicillamine enkephalin; DAMGO, D-Ala², N-Me-Phe⁴, Gly⁵-ol; CNS, central nervous system

2.4. Immunocytochemistry

Immunohistochemical analysis was performed as previously reported [5]. Adult Wistar rats weighing 250–300 g were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer under ether anesthesia. The specimens were immersed in 30% sucrose/0.1 M phosphate buffer. Sections were cut at a thickness of 20 μ m with a cryostat and mounted onto gelatin-coated slide glasses. After incubation in PBS containing 0.05% Triton X-100, the sections were incubated in 0.3% H_2O_2 /methanol for the inhibition of the endogenous peroxidase activities. The sections were then exposed to 5% normal goat serum for 30 min to block non-specific staining. Subsequently, the sections were incubated with the primary antibody at a final concentration of 1 μ g/ml, followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 1 h, washed 6 times with PBS, and placed in the avidin-biotin-HRP complex (ABC kit, Vector Laboratories). The sections were then washed 3 times with PBS and treated with 0.01% 3',3'-diaminobenzidine tetrahydrochloride, Tris-HCl pH 7.5 and 0.002% H_2O_2 . In control experiment, sections were incubated with the primary antibody pre-absorbed with 10 μ g/ml antigen peptide.

2.5. Expression of rat oatp2 in *Xenopus* oocytes

The cDNA encoding rat oatp2 was linearized and the capped cRNA was transcribed *in vitro* with T7 RNA polymerase (Stratagene). *Xenopus laevis* oocytes were prepared as described previously [1]. After an overnight incubation at 18°C, healthy defolliculated oocytes were microinjected with 10 ng of rat oatp2 cRNA and subsequently cultured for 48–72 h in a modified Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES pH 7.6). Uptake of radio-labeled chemicals was measured in a medium containing 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES pH 7.5. Water-injected oocytes were used as controls. Five to nine oocytes were prewashed in the uptake medium and incubated at room temperature in 100 μ l of the same medium containing radio-labeled substrate at the indicated concentration for 1 h. Uptake was terminated by addition of 1 ml of ice-cold uptake buffer and the oocytes were washed 3 times with 3 ml of ice-cold uptake buffer. Each oocyte was dissolved in 500 μ l of 10% SDS and 4 ml of scintillation fluid (Packard), and the oocyte-associated radioactivity was counted in a Packard Tri-Carb 2100 liquid scintillation counter (Packard). The statistical significance was tested by unpaired *t*-test.

3. Results and discussion

3.1. Western blot analysis

Western blot analysis was performed after 10% SDS-polyacrylamide gel electrophoresis of the crude membranes (10 μ g). As shown in Fig. 1, the anti-oatp2 antibody recognized a faint band in the brain (\sim 76 kDa) and a closely spaced doublet of proteins in the liver, with an apparent molecular size of \sim 74 kDa and \sim 76 kDa. These oatp2-positive bands

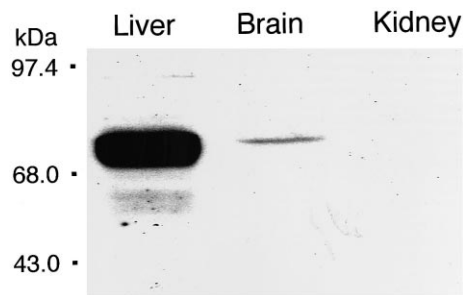


Fig. 1. Western immunoblot of rat oatp2. Western blot analyses of rat liver membranes were performed with the affinity-purified antibody against rat oatp2. 10 μ g of membrane was separated on 10% SDS-PAGE gel under denaturing conditions and subjected to immunoblotting with the affinity-purified antibody.

disappeared completely when the antibody was preabsorbed with the antigen peptide (data not shown), suggesting the specificity of the detected bands. On the other hand, no immunoreactivity was detected in the kidney. These data are consistent with our Northern blot analysis [1], and also support that oatp2 is not expressed in the kidney as much as reported by Noé et al. [2]. In the liver, the oatp2 antibody detected two bands (Fig. 1); one is located at the same size as that in the brain and the other is relatively low (detected by the short exposure, data not shown). Because the predicted apparent molecular size of rat oatp2 is 73.2 kDa [1,2] and there are six putative glycosylation sites in its structure, the existence of two bands may represent different manners of glycosylation between the brain and liver.

In our experiment, oatp2 immunoreactivity was clearly detected using 10 μ g of crude membrane (Fig. 1). On the other hand, Western blot analysis detected faint oatp1 immunoreactivity (\sim 80 kDa) when the crude membrane of the liver (20 μ g) was used [6]. Comparing these data, it is suggested that, in the liver, oatp2 is expressed more highly than oatp1 at the protein level.

3.2. Localization of oatp2 in rat liver

By immunohistochemical analysis, the oatp2 immunoreactivity was significantly detected around the central vein in the liver (Fig. 2a). At high magnification, oatp2 immunoreactivity was observed at the basolateral (sinusoidal) membrane of the hepatocyte that faces the sinusoid (Fig. 2b, indicated by arrowhead). In contrast, no signal was detected intracellularly

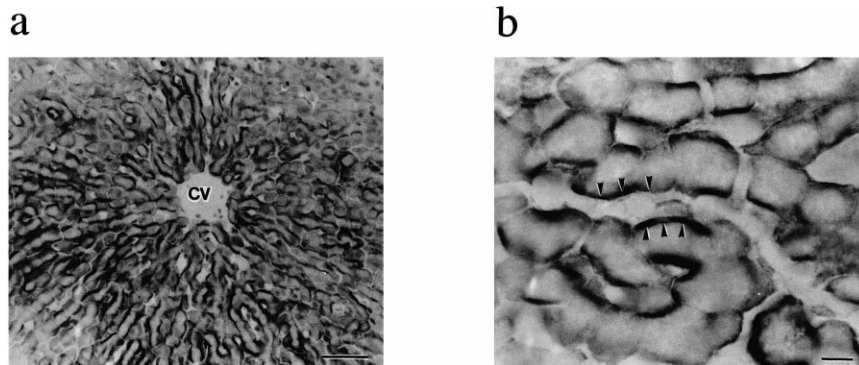


Fig. 2. Immunohistochemical localization of oatp2 in rat liver. a: Note that oatp2 immunoreactivity was seen in the liver around the central vein (CV). b: At a higher magnification, immunoreactivity was observed in the basolateral membranes of the hepatocytes (arrowhead). Magnification view of the liver, 750 \times (a), 150 \times (b). Scale bar, 50 μ m (a) and 10 μ m (b).

Table 1
Uptake of compounds by oatp2-expressing oocytes

Tracer (μM)	Tracer uptake (pmol/oocyte)		Uptake ratio (oatp2/H ₂ O)	
	H ₂ O	oatp2		
Thyroxine (1)	0.29 \pm 0.01	3.8 \pm 1.0	13	**
Taurocholate (12)	0.06 \pm 0.02	3.89 \pm 0.18	65	**
Estradiol 17 β -glucuronide (0.76)	0.18 \pm 0.01	0.60 \pm 0.07	3.3	**
Estrone sulfate (0.76)	0.10 \pm 0.02	0.97 \pm 0.11	9.5	**
DHEAS (0.44)	0.027 \pm 0.002	1.7 \pm 0.14	61	**
Digoxin (2.7)	0.16 \pm 0.11	4.2 \pm 0.45	26	**
DPDPE (12)	0.018 \pm 0.004	1.3 \pm 0.3	72	**
Leu-enkephalin (1)	0.31 \pm 0.02	0.43 \pm 0.02	1.4	*
Biotin (0.69)	0.027 \pm 0.001	0.045 \pm 0.008	1.7	*
[D-Ala ² ,Glu ⁴]Deltorphin (0.76)	0.12 \pm 0.01	0.15 \pm 0.02	1.3	
DAMGO (0.76)	0.01 \pm 0.001	0.013 \pm 0.002	1.3	
Aldosterone (0.48)	0.74 \pm 0.02	0.76 \pm 0.04	1.0	
Estradiol (1)	6.2 \pm 0.57	6.35 \pm 0.45	1.0	
Testosterone (0.40)	2.2 \pm 0.1	2.06 \pm 0.13	0.9	

The uptake experiments were performed at the concentrations indicated. The oatp2/water uptake ratio (%) is shown. Values are means \pm S.E.M. of 8–15 oocyte determinations. Significance between water-injected and oatp2-cRNA-injected oocytes was determined by unpaired *t*-test (**P* < 0.05 and ***P* < 0.01).

or at the canalicular membrane. This immunoreactivity was specific to oatp2, because preabsorption of the antibody with excess peptide immunogen resulted in the complete abolition of the immunostaining described above (data not shown). In the liver, oatp1, another member of the oatps, is expressed at the basolateral membrane of the hepatocyte [6]. GLUT-2 immunoreactivity is also distributed predominantly in the periportal and the perivenous zones of the hepatocytes [7]. It is, thus, suggested that oatp2 would be involved in the clearance mechanisms at the basolateral membrane of hepatocytes, taking up its specific substrates from the circulation (see Section 3.3).

3.3. Expression of oatp2 in *Xenopus* oocytes

In order to study the substrate selectivity of oatp2, uptake experiments were performed (Table 1). As reported previously, oatp2 transports taurocholate, digoxin thyroid hormones (T₄, T₃) and conjugated steroids (estradiol 17 β -glucuronide and estrone sulfate), in a saturable manner [1,2]. As shown in Table 1, the oatp2-expressing oocytes significantly transported radiolabeled taurocholate, digoxin and thyroxine. The oatp2-expressing oocytes also significantly transported the conjugated steroid dehydroepiandrosterone sulfate (DHEAS). In contrast, unconjugated steroids such as aldosterone, estradiol and testosterone were not transported. DHEAS is the most abundant product of the adrenal cortex and provides a universal precursor of further androgenic and estrogenic steroids in peripheral tissues. In the liver, DHEAS is transported by a saturable carrier-mediated process in vivo [8]. In the central nervous system (CNS), it has also been shown that DHEAS is transported into the CNS, and increases neural excitability [9], improves memory [10] and enhances the survival of neurons in culture [11]. Furthermore, Kullak-Ublick et al. reported that human OATP is one of the genes responsible for transporting DHEAS in the brain [12]. Therefore, oatp2 may be another candidate transporter which is responsible for taking up DHEAS in the brain and liver.

In the brain, some peptides can enter the CNS through both non-saturable and saturable transporting mechanisms [13]. Our recent study in situ hybridization revealed that oatp2 mRNA is widely expressed in the rat brain (especially

in the choroid plexus, hippocampus and cerebellum) [1]. Because oatp2 is also detected in the rat brain at the protein level (Fig. 1), we studied whether oatp2 transports peptides. Among the peptides tested, the δ_1 opioid receptor agonist [D-Pen²,D-Pen⁵]enkephalin (DPDPE) was transported significantly more than the other opioid receptor agonists Leu-enkephalin, [D-Ala²,Glu⁴]deltorphin (a selective δ_2 agonist) and D-Ala²,N-Me-Phe⁴,Gly⁵-ol (DAMGO, a selective μ opioid receptor agonist) (Table 1). DPDPE is an enzymatically stable δ_1 opioid receptor-selective peptide which produces analgesia [14,15]. The passage of DPDPE across the blood-brain and the blood-cerebrospinal fluid barriers has been well investigated [16–19]. Thomas et al. further revealed a saturable DPDPE uptake into the brain that followed Michaelis-Menten kinetics [20]. In the liver, the existence is also suggested of a facilitated transport system for DPDPE which may mediate the rapid disappearance from the systemic circulation [21]. Thus, oatp2 appears to be one of the molecules that is involved in the uptake of DPDPE in vivo.

Leu-enkephalin was weakly transported by the oatp2-expressing oocytes. Although carrier-mediated mechanisms for Leu-enkephalin across the blood-brain barrier have been predicted [22], the uptake of DPDPE into the brain was not inhibited by Leu-enkephalin [16] and [D-Ala²,Glu⁴]deltorphin [15]. Because oatp2 transports both DPDPE and Leu-enkephalin, the existence of additional candidate transporter(s) or mechanisms for such peptides in the CNS is suggested.

In our experiment, oatp2 transported biotin significantly (Table 1). Recently, a rat cDNA encoding a 12 transmembrane type transporter which transports biotin and pantothenate in a Na⁺-dependent manner has been isolated [23]. Thus, the present results raise the possibility that multiple transporting mechanisms would be responsible for the transport of biotin.

So far, multispecificity of the oatp family substrate has been reported [1,2,24]. Both rat oatp1 and human OATP transport taurocholate, 17- β -estradiol glucuronide and estrone sulfate with significant differences in initial uptake rate and apparent *K_m* values [2,23,24]. In addition, no reports on human OATP- or oatp1-mediated uptake of substrates described in this report (i.e. thyroxine, DPDPE, Leu-enkephalin, biotin) have

been made. Thus, further pharmacological characterization of the substrate specificities among the oatp family should provide new insight into bile acid formation and greater understanding of the multispecificity of the oatp family.

In conclusion, oatp2 is revealed to be expressed in the brain and liver at the protein level. In the liver, oatp2 is located at the basolateral membrane of the hepatocytes, suggesting the facilitated transport of thyroid hormones, various organic anions, conjugated steroids and neuropeptides.

Acknowledgements: We thank Dr. Kazuo Nunoki for critical reading of the manuscript. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Yamanouchi Foundation for Research on Metabolic Disorders, the Nishimiya Foundation, the Japan Research Foundation for Clinical Pharmacology and the Inamori Foundation.

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