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Identification of organic anion transporting polypeptide 4 (Oatp4) as a major full-length isoform of the liver-specific transporter-1 (rlst-1) in rat liver

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Abstract A novel organic anion transporting polypeptide (Oatp)4[1] was isolated from rat liver that is 35 amino acids longer than the reported rat liver specific organic anion transporter (rlst)-1 and exhibits a 64% amino acid sequence identity with the human OATP-C (LST-1/OATP2; gene symbol *SLC21A6*). When expressed in *Xenopus laevis* oocytes, Oatp4 *(Slc21a10)* mediated polyspecific uptake of a variety of organic anions including taurocholate ($K_m \sim 27$ μM), bromosulfophthalein ($K_m \sim 1.1$ μM) and steroid conjugates. Based on nuclease protection analysis Oatp4 appears to be the predominant transcript in rat liver indicating that rlst-1 plays a minor role in overall hepatic organic anion uptake.

Key words: Liver transport; Bile salt; Sulfobromophthalein; Steroid conjugate; Thyroid hormone; Leukotriene C4

1. Introduction

The mammalian liver removes a wide variety of endogenous and exogenous (i.e. xenobiotic) amphipathic organic compounds from blood plasma. The first step of this hepatic solute clearance process is mediated to a large extent by active transport systems at the sinusoidal (basolateral) plasma membrane of hepatocytes. They include the Na^+–taurocholate cotransporting polypeptide (rat: Ntcp; human: NTCP) for Na^+-dependent and the organic anion transporting polypeptides (rat: Oatps; human: OATPs) for Na^+-independent uptake of bile salts and non-bile salt organic anions, respectively [1]. Several rat and human OATPs have been identified in various tissues and shown to represent polyspecific organic solute carriers with mostly overlapping and partially distinct substrate specificities [2,3]. Oatp/OATP family members include the closely related (amino acid sequence identities ≥67%) Oatp1 [4], Oatp2 [5], Oatp3 [6], rat kidney organic anion transporting polypeptide (OAT-K)1 [7], OAT-K2 [8] and the human OATP-A (previously called OATP) [9], the more distantly related rat (PGT) [10] and human (hPGT) [11] prostaglandin transporters (amino acid sequence identities of 38 and 39% to Oatp1 and OATP-A, respectively) and the recently cloned rat (rlst-1) [12] and human (LST-1) [13] liver specific transporters (amino acid sequence identities of 44% to Oatp1 and OATP-A). LST-1 has been initially deposited as OATP-C in the GenBank (AB026257) and has also been called OATP2 [14,15]. It should, however, be realized that the human OATP2 does not represent the direct orthologue of the rat Oatp2. In order to avoid false associations of non-orthologous proteins between species and to keep the functionally based family designation 'OATP', we prefer to provisionally follow the alphabetical GenBank nomenclature and use OATP-C rather than LST-1/OATP2 in this study.

Among Oatps/OATPs highly expressed in the liver (e.g. Oatp1, Oatp2, OATP-C, rlst-1), the recently identified rat liver specific transporter (rlst-1) has been interpreted as the rat orthologue of the human OATP-C [12,13]. However, rlst-1 differs from OATP-C by a deletion of 35 amino acids [12,13] resulting in an 11 instead of the characteristic 12 predicted transmembrane domain topology of the other members of the Oatp/OATP family. Furthermore, the transport activity of rlst-1 is restricted to taurocholate whereas OATP-C exhibits a broad organic anion transport activity [12,13]. In this study we report the identification of a full-length rlst-1 isoform, which we call Oatp4 based on the previously established consecutive numbering principle of rat Oatps. This Oatp4 exhibits the expected 12 transmembrane domain topology of the other family members and demonstrates a similar polyspecific transport activity as OATP-C. Furthermore, expression of Oatp4 in rat liver is shown to be considerably more abundant than rlst-1 indicating that Oatp4 plays a major role in the hepatic uptake of organic anions, whereas rlst-1 appears to be a minor splice-variant of Oatp4 with a uncertain physiologic function.

2. Materials and methods

2.1. Materials

[6,7-^{3}H(N)]Estrone-3-sulfate (53 Ci/mmol), [6,7-^{3}H(N)]estradiol-17β-glucuronide (44 Ci/mmol), [^{3}H(G)]taurocholate (2.0 Ci/mmol),

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[1] Sequence deposition: The EMBL/GenBank accession number of the Oatp4 sequence is AJ271682.

Abbreviations: BSP, sulfobromophthalein; LST-1(OATP-C, OATP2), human liver specific transporter-1; Oatp, rat organic anion transporting polypeptide; OATP, human organic anion transporting polypeptide; OAT-K, rat kidney organic anion transporting polypeptide; PGT, prostaglandin transporter; rlst-1, rat liver specific transporter-1

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[^{3}H(N)]prostaglandin E_2 (200 Ci/mmol), [^{3}H(N)]leukotriene C_4 (158 Ci/mmol), L-[^{125}I]triiodothyronine (T3, 779 Ci/mmol), L-[^{125}I]thyroxine (T4, 969 Ci/mmol), [^{3}H]dehydroepiandrosterone sulfate (60 Ci/mmol) and [^{3}H]digoxin (19 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). [^{35}S]Sulfobromophthalein (BSP) (9 Ci/mmol) was synthesized as described [16].

2.2. Animals

Female *Xenopus laevis* were purchased from the African *Xenopus* facility c.c., Noordhoek, R. South Africa. Male Sprague–Dawley rats were from RCC, Füllinsdorf, Switzerland.

2.3. RT-PCR with primers derived from rlst-1 and flanking the putative missing transmembrane domain of rlst-1

Total rat liver RNA was prepared as described [17] and mRNA isolated using the PolyATtract mRNA isolation system (Promega). cDNA was synthesized from rat liver mRNA using the Superscript kit (Gibco BRL), and PCR was performed using forward (5′-CATACCTACTATGGCAACTGC-3′) and reverse (5′-GTTGGTTTTGCATTTGTATCT-3′) primers. Cycling conditions on a Robocycler (Stratagene) were: one cycle of 94°C/2 min followed by 30 cycles of 94°C/45 s, 48°C/45 s, 72°C/45 s and a final elongation of 72°C/5 min. The resulting amplicons (440 bp for Oatp4 and 335 bp for rlst-1) were extracted from a 3% TAE–agarose gel, subcloned into the pGEM-T vector (Promega) and sequenced.

2.4. Isolation of Oatp4 open reading frame by RT-PCR

cDNA was synthesized from rat liver mRNA using the Superscript kit (Gibco BRL). PCR amplification was performed under the following conditions: the forward primer containing a *Nco*I restriction site 5′-GCCGCCATGGACCACACTCAGCAGTCAAG-3′, the reverse primer containing a *Hin*dIII restriction site 5′-GTACGTAAGCTTAAAGAGGTGTTTCATTG-3′, one cycle of 94°C/2 min followed by 30 cycles of 94°C/45 s, 53°C/45 s, 72°C/3 min and a final elongation of 72°C/10 min. The resulting amplicon was isolated from a 1% TAE–agarose gel and cut for 2 h at 37°C with the restriction endonucleases *Nco*I and *Hin*dIII. After another gel purification the amplicon was inserted into a *X. laevis* expression vector and sequenced on both strands. The *X. laevis* expression vector was constructed by removing the open reading frame of the rat Oatp1 with *Nco*I and *Hin*dIII. Thus any open reading frame cloned into this vector will have the 3′ trailer sequence of Oatp1 for efficient stability and expression of its cRNA in *X. laevis* oocytes.

2.5. Construction and screening of a rat liver cDNA library

Rat liver mRNA was prepared, size-fractionated and analyzed by Northern blotting as described [17] using the *Nco*I/*Hin*dIII fragment of Oatp4. cDNA was synthesized from a mRNA size class that was enriched for Oatp4/rlst-1 using the Superscript kit (Gibco BRL) and ligated unidirectionally into the *Sal*I and *Not*I sites of plasmid pSport1 (Gibco BRL). Recombinant plasmids were introduced into *Escherichia coli* WM1100 by electroporation (Gene Pulser; Bio-Rad) resulting in a library of 1.5×10^5 clones. Pools of 1500 clones were grown over night on agar plates in the presence of ampicillin and replicated onto nylon filters (Hybond N, Amersham-Pharmacia). Hybridization screening was performed in the ExpressHyb hybridization solution (Clontech) using a ^{32}P-labelled Oatp4 specific cDNA probe (*Nco*I/*Hin*dIII fragment). The filters were washed twice for 15 min at room temperature with 2×SSC/0.05% SDS and twice for 15 min at 55°C with 0.1×SSC/0.1% SDS. After exposure over night on an X-ray film (Biomax, Kodak) positive single colonies were isolated and identified as full-length Oatp4 by sequencing both strands.

2.6. Uptake experiments in X. laevis oocytes

Oatp4 cRNA was transcribed in vitro from *Not*I-linearized cDNA using the mMESSAGE mMACHINE T7 kit (Ambion). *X. laevis* oocytes were prepared [18] and injected either with 50 nl water or with 50 nl of a 0.1 ng/nl cRNA solution. For optimal protein expression the oocytes were kept 3 days in culture at 18°C with daily changes of the Barth's solution. Uptake of radiolabelled substrates was measured for 10 min at 25°C in 100 μl uptake solution (100 mmol/l choline chloride, 2 mmol/l KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, and 10 mmol/l HEPES adjusted to pH 7.5 with Tris) as described [3].

2.7. RNase protection assay

After linearization of the 440 bp Oatp4-specific RT-PCR amplicon with *Nco*I, antisense RNA was synthesized using the SP6 MAXIscript kit (Ambion). Rat liver total RNA was then used with the HybSpeed RPA kit (Ambion) according to the instructions of the manufacturer. For each sample the same amount of radioactivity (6×10^4 cpm) was used. The protected bands were visualized on an X-ray film after separation on a 5% polyacrylamide/8 M urea gel.

3. Results and discussion

To investigate whether a full-length polyspecific OATP-C orthologue is present in rat liver, we first performed RT-PCR with primers derived from the flanking regions of the putatively missing transmembrane domain of rlst-1. Two cDNA fragments of 335 and 440 bp were obtained. Subcloning and sequencing of the two fragments showed that the 335 bp amplicon corresponded to rlst-1 and the 440 bp to a putatively longer isoform of rlst-1. In order to amplify both the longer new clone and rlst-1, a PCR was performed from rat liver cDNA using primers spanning the whole open reading frame of rlst-1. After cloning of the resulting amplicon into the *X. laevis* expression vector (see Section 2.4), we obtained a functionally active single cDNA clone containing an open reading frame of 2061 bp encoding a 687 amino acid polypeptide. The latter was used as a probe to isolate the native cDNA from a size fractionated rat liver cDNA library. The resulting cDNA clone contained in addition to the open reading frame 115 bp 5′ leader and 1031 bp 3′ trailer sequences. The derived amino acid sequence of this clone was identical to rlst-1 with the exception of an additional 35 amino acid insert after amino acid 406 (Fig. 1A). This new Oatp is called Oatp4, since previous rat members of the Oatp family, that have been isolated from liver and brain, have been consecutively num-

Table 1
Substrate uptake activities into Oatp4-cRNA (Oatp4) and water (H_2O) injected *X. laevis* oocytes

Substrates	Oatp4[a] (fmol/oocyte×min)	H_2O[a] (fmol/oocyte×min)	Oatp4/H_2O (uptake ratios)
Taurocholate (5 μM)	18.3 ± 4.1	2.9 ± 0.7	6.3
BSP (0.72 μM)	61.7 ± 18.6	3.6 ± 0.7	17.1
Estrone-3-sulfate (0.19 μM)	4.7 ± 1.3	0.28 ± 0.02	16.8
Estradiol-17β-glucuronide (0.23 μM)	9.2 ± 2.3	0.40 ± 0.09	23.0
Dehydroepiandrosterone sulfate (0.17 μM)	6.5 ± 1.1	0.20 ± 0.03	32.5
Prostaglandin E_2 (0.2 μM)	0.26 ± 0.04	0.07 ± 0.01	3.6
Leukotriene C_4 (0.005 μM)	0.05 ± 0.01	0.015 ± 0.003	3.3
T3 (0.013 μM)	0.32 ± 0.08	0.17 ± 0.02	1.9
T4 (0.010 μM)	0.20 ± 0.04	0.09 ± 0.02	2.2
Digoxin (0.6 μM)	1.8 ± 0.3	1.8 ± 0.4	1.0

[a]Values represent means ± S.D. of 8–10 uptake determinations. Stage V–VI oocytes were injected either with 5 ng cRNA or with equivalent volume (50 nl) of water. Uptakes of radiolabelled substrates were measured for 10 min at 25°C in 100 μl uptake solution.

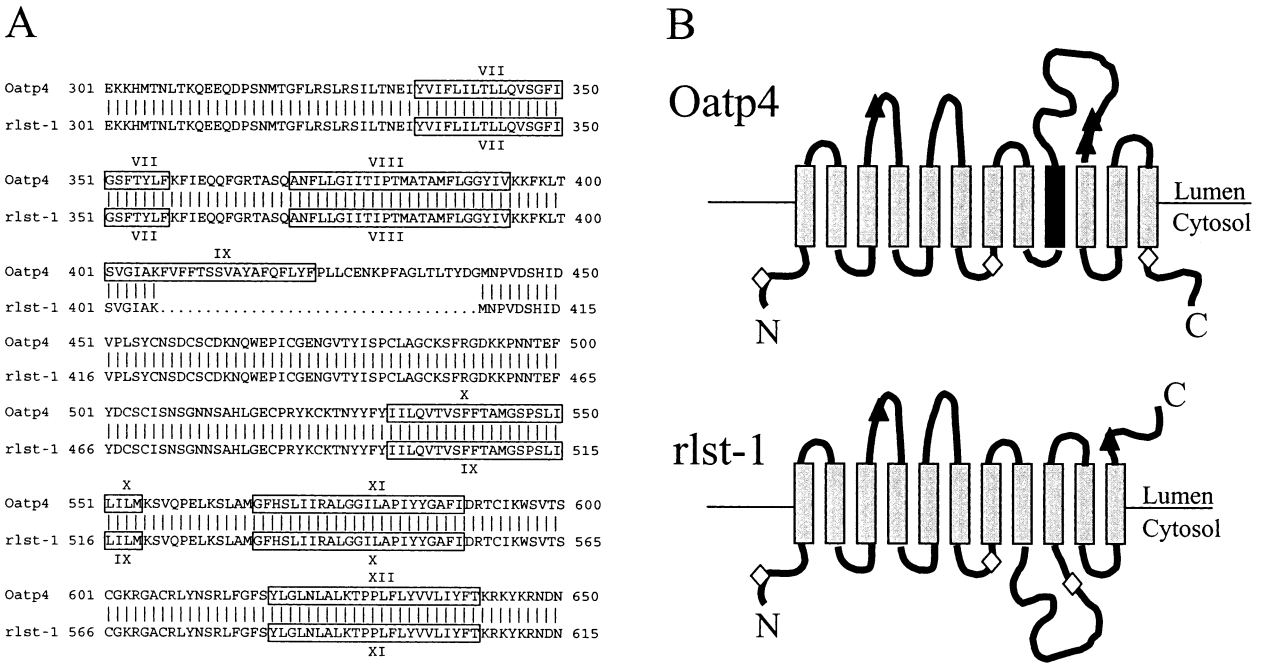

Fig. 1. Comparison of Oatp4 and rlst-1. A: Amino acid alignment of Oatp4 and rlst-1 with putative transmembrane domains. Since the two proteins are identical except for the gap of 35 amino acids, only this divergent part of the alignment is shown. B: Comparison of the predicted topologies of Oatp4 and rlst-1. The additional 35 amino acids of Oatp4 contain a potential transmembrane helix which led to an inverted topology of the second half of Oatp4 as compared to rlst-1. (▲), potential *N*-glycosylation sites, (◇), potential PKC phosphorylation sites. Transmembrane domains were predicted using the neural network at the Centre for Biological Sequence Analysis, Department of Biotechnology of the Technical University of Denmark (http://www.cbs.dtu.dk/services/TMHMM-1.0/). Glycosylation and phosphorylation sites were detected using the PROSITE scan at http://www.expasy.ch/tools/scnpsit1.html.

bered Oatp1, 2 and 3 [4–6]. Based on hydrophobicity analysis, the additional amino acids of Oatp4 complement the transmembrane domain IX and thus indicate a similar 12 transmembrane topology of Oatp4 as for the other members of the Oatp/OATP family (Fig. 1B) [19]. Thus, the N- and C-terminal ends of Oatp4 both lay intracellularly and the 108 amino acid stretch between the transmembrane domains IX and X containing two potential glycosylation sites is shifted extracellularly (Fig. 1B). In addition, Oatp4 contains three potential PKC-phosphorylation sites in the cytosolic hydrophilic loops (Fig. 1B) [20,21]. The overall amino acid sequence identity of Oatp4 is 64% with the human OATP-C, 40–44% with rat Oatp1, Oatp2, Oatp3, OAT-K1 and OAT-K2 and 33% with PGT. Thus, and similar to rlst-1 [12], Oatp4 is closely related to OATP-C and positions between the rat Oatp1-3/OAT-K1,2 and the rat PG transporter.

To investigate whether the complemented amino acid sequence and the predicted change in the topology of Oatp4 as compared to rlst-1 is associated with additional new transport functions, we performed uptake studies in Oatp4-cRNA

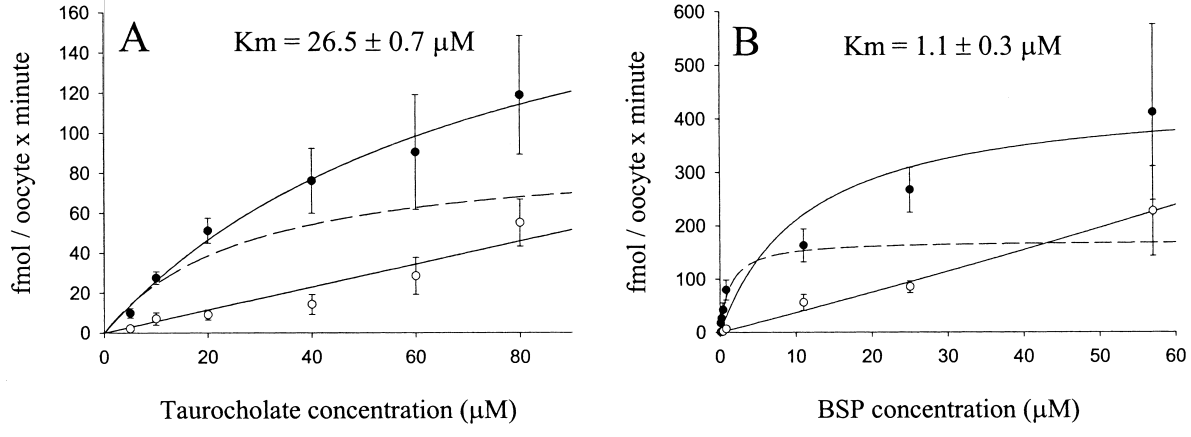

Fig. 2. Kinetics of Oatp4-mediated taurocholate (A) and BSP (B) uptake in *X. laevis* oocytes. Uptake measurements were performed in the presence of increasing substrate concentrations in Oatp4-cRNA (●) and water-injected (○) oocytes. Individual values are given as means ± S.D. of 8–10 uptake measurements. Lines were fitted with by non-linear regression analysis using the Systat 8.0 program assuming Michaelis–Menten kinetics. The dashed line represents the difference between uptakes into cRNA and water injected oocytes and was the basis for the estimation of the indicated apparent K_m values.

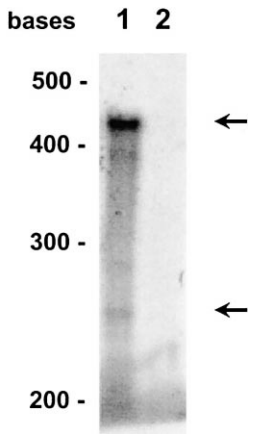

Fig. 3. RNase protection assay with total rat RNA. The RNA was protected using the Oatp4 specific 440 bp RT-PCR amplicon. Protection of Oatp4 should yield a fragment of 440 bases while protection of rlst-1 should yield two fragments of 256 and 79 bases, respectively. Lane 1: protection of 10 μg total liver RNA in the presence of 40 μg yeast tRNA. Lane 2: protection of 50 μg yeast tRNA. Arrows point to the protected fragments of 440 and 256 bases, respectively.

injected *X. laevis* oocytes. In contrast to rlst-1, that has been shown to mediate only Na^+-independent taurocholate uptake with an apparent K_m value of ~9.5 μM [12], Oatp4 expression induced uptake of a variety of organic anions such as taurocholate (K_m ~26.5 μM), BSP (K_m ~1.1 μM), estrone-3-sulfate, estradiol-17β-glucuronide, dehydroepiandrosterone sulfate, and to a lesser degree also prostaglandin E_2, leukotriene C_4 and the thyroid hormones T3 and T4 (Table 1 and Fig. 2). These transport properties of Oatp4 are very similar to the transport characteristics of the human OATP-C [13–15], but also of rat Oatp1 and Oatp2 with the notable exceptions of BSP (not transported by Oatp2) and digoxin (high affinity substrate of Oatp2, but not transported by Oatp4) (Table 1) [3]. Although these data support the close relationship between rat Oatp4 and human OATP-C, it remains uncertain, based on the 64% amino acid sequence identity, whether Oatp4 represents a direct orthologue of OATP-C.

Finally, we wondered about the expression levels of Oatp4 mRNA as compared to rlst-1 in rat liver. As illustrated in Fig. 3, a strong RNase protection signal was obtained for the expected Oatp4-specific 440 base fragment, whereas RNase protection for the expected rlst-1 256 base fragment was minimal. These data indicate predominant expression of Oatp4 in rat liver, whereas rlst-1 might represent a minor splice-variant of Oatp4 with a transport function restricted to taurocholate. Since all liver Oatps/OATPs, including OATP-C [15], have been localized so far to the basolateral membrane of hepatocytes, the data support an important role of Oatp4, together with Oatp1 and Oatp2 [3], for organic anion uptake into rat hepatocytes.

In conclusion, we have identified a full-length Oatp of rlst-1 in rat liver. This so called Oatp4 exhibits a similar predicted 12 transmembrane domain topology as the other members of the *Oatp/OATP* gene family (symbol *SLC21A*), are strongly expressed in rat liver and exhibit polyspecific organic anion transport activities. While these results indicate that Oatp4 contributes significantly to organic anion transport in rat liver, its exact hepatocellular localization and its distinct transport properties in relation to Oatp1 and Oatp2 remain to be investigated.

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