Effect of antisense oligonucleotides on the expression of hepatocellular bile acid and organic anion uptake systems in *Xenopus laevis* oocytes

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A Na^+-dependent bile acid (Na^+/taurocholate co-transporting polypeptide; Ntcp) and a Na^+-independent bromosulphophthalein (BSP)/bile acid uptake system (organic-anion-transporting polypeptide; oatp) have been cloned from rat liver by using functional expression cloning in *Xenopus laevis* oocytes. To evaluate the extent to which these cloned transporters could account for overall hepatic bile acid and BSP uptake, we used antisense oligonucleotides to inhibit the expression of Ntcp and oatp in *Xenopus laevis* oocytes injected with total rat liver mRNA. An Ntcp-specific antisense oligonucleotide co-injected with total rat liver mRNA blocked the expression of Na^+-dependent taurocholate uptake by approx. 95%. In contrast, an oatp-specific antisense oligonucleotide when co-injected with total rat liver mRNA had no effect on the expression of Na^+-dependent taurocholate uptake, but it blocked Na^+-independent uptake of taurocholate by approx. 80% and of BSP by 50%. Assuming similar expression of hepatocellular bile acid and organic anion transporters in *Xenopus laevis* oocytes, these results indicate that Ntcp and oatp respectively represent the major, if not the only, Na^+-dependent and Na^+-independent taurocholate uptake systems in rat liver. By contrast, the cloned oatp accounts for only half of BSP transport, suggesting that there must be additional, non-bile acid transporting organic anion uptake systems in rat liver.

INTRODUCTION

Amphipathic organic anions such as bile acids, bilirubin and bromosulphophthalein (BSP) are taken up from portal blood plasma into hepatocytes across the basolateral plasma membrane by various carrier-mediated transport processes [1–10]. Candidate proteins in the molecular mass range 48–54 kDa have been identified and labelled by using photoaffinity and immunological techniques [11–16], but except for microsomal epoxide hydrolase, which has been proposed to be targeted as a special isoform to the basolateral membrane where it could be involved in Na^+-dependent uptake of bile acids [17], none of these proteins has been characterized at the molecular level.

Two hepatocellular bile acid transporters have been cloned by means of functional expression cloning in *Xenopus laevis* oocytes. The first system represents a 51 kDa Na^+-dependent taurocholate co-transporting polypeptide (Ntcp) [18,19] that transports physiological bile acids such as cholate, taurocholate, taurochenodeoxycholate and tauroursodeoxycholate in a Na^+-dependent way. The second system represents a Na^+-independent organic-anion-transporting polypeptide (oatp) [20] that mediates Na^+-independent transport of a variety of amphipathic substrates, including bile acids and BSP [20,21].

Because additional candidate proteins have been proposed [4,9,14,17], we investigated the physiological importance of Ntcp and oatp respectively, for overall bile acid and organic anion uptake into rat hepatocytes. For this purpose total rat liver mRNA was injected into *Xenopus laevis* oocytes and expression of Ntcp and oatp was selectively blocked by specific antisense oligonucleotides. The results suggest that most if not all of the bile acid uptake into rat liver is mediated by Ntcp and oatp. In contrast, oatp is only responsible for about half of the BSP uptake, suggesting the presence of additional, non-bile acid organic-anion transporters in rat liver.

MATERIALS AND METHODS

Materials

[G-^{3}H]Taurocholic acid (2.0 Ci/mmol) was purchased from Du Pont–New England Nuclear (Boston, MA, U.S.A.); [^{35}S]BSP, prepared at a specific radioactivity of 3–4 Ci/mmol by the method of [22], was kindly provided by A. W. Wolkoff of Albert Einstein College of Medicine (Bronx, New York, NY, U.S.A.).

Isolation and synthesis of RNA

Total rat liver RNA was prepared as described [23] and mRNA was isolated with the PolyATtract mRNA isolation system (Promega Corp., Madison, WI, U.S.A.).

Oligonucleotides

Unmodified sense or antisense oligonucleotides of Ntcp and oatp were purchased from Mycrosynth (Balgach, Switzerland). They had the following sequences: Ntcp-sense (nucleotides 593–612), 5′-GGCATTATGATATCACTAGT-3′; Ntcp-antisense1 (nucleotides 543–562), 5′-ATCGTAGATGCCTTTG-CTGT-3′; Ntcp-antisense2 (nucleotides 815–834), 5′-TAAC-CCATCAGAAAGCCAGA-3′; oatp-sense (nucleotides 522–537), 5′-CCAGCAGAATGTGTGA-3′; oatp-antisense1 (nucleotides 555–570), 5′-TTACACATATCCACAT-3′; oatp-

Abbreviations used: BSP, bromosulphophthalein; cRNA, RNA synthesized *in vitro*; Ntcp, Na^+/taurocholate co-transporting polypeptide; oatp, organic-anion-transporting polypeptide.

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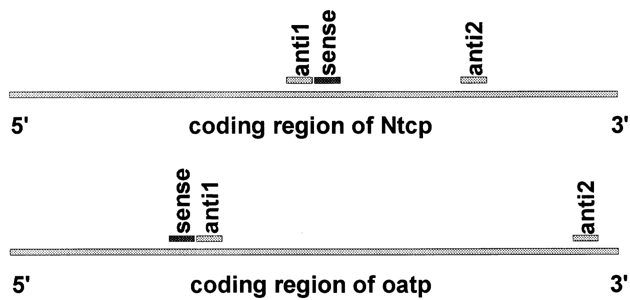

Figure 1 Schematic localization of the various sense and antisense oligonucleotides within the coding regions of Ntcp and oatp

For the exact localization and sequence of the oligonucleotides see the Materials and methods section.

antisense2 (nucleotides 1919–1934), 5′-GGCAGGCAGATAGCTT-3′. The exact positions of the sense and antisense oligonucleotides are illustrated in Figure 1.

Injection of *Xenopus laevis* oocytes and transport assays

Xenopus laevis oocytes were prepared as described [24] with the following modifications. The follicle cell layer was removed from the oocytes by incubation at room temperature in Ca^{2+}-free OR-2 [24] solution supplemented with 2 mg/ml collagenase type D (Boehringer Mannheim). After 45 min the collagenase solution was changed and the incubation was continued with microscopic inspections of the oocytes until the digestion was completed (usually after 15 to 30 min). After several washes in modified Barth's solution, stage V and VI oocytes were selected and incubated overnight at 18 °C. After cooling on ice, 50 nl of a solution containing 50 mM NaCl, 2.5–5 ng of oligonucleotides and either 0.5 ng of RNA synthesized *in vitro* (cRNA) or 50 ng of total rat liver mRNA was injected into *Xenopus laevis* oocytes. The oocytes were incubated for 1–5 days at 18 °C and the expression of taurocholate and BSP uptake activities were measured at 25 °C in a medium containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris, pH 7.5, and 10 μM [^{3}H]taurocholate or 2 μM [^{35}S]BSP as described [24,25].

Statistical analysis

All values are given as means ± S.E.M. Differences between mean values were assessed by Kruskal–Wallis ANOVA, followed by the Wilcoxon/Mann–Whitney test. $P < 0.05$ was considered significant.

RESULTS

Oligonucleotide specificity

As demonstrated in Table 1, neither Ntcp-sense nor oatp-antisense2 oligonucleotides exerted any inhibitory effects on expression of Na^+-dependent taurocholate uptake in oocytes injected with Ntcp cRNA. In contrast, both Ntcp antisense oligonucleotides completely inhibited the functional expression of Ntcp. Furthermore, in oocytes injected with oatp cRNA, taurocholate uptake was affected by neither the oatp-sense nor the Ntcp-antisense2 oligonucleotides, whereas both oatp antisense oligonucleotides almost completely inhibited expression of oatp-mediated taurocholate uptake. These results demonstrate specific inhibition of the expression of Ntcp- and

Table 1 Effects of sense and antisense oligonucleotides of Ntcp and oatp in cRNA-injected oocytes

Oocytes were either not injected or injected with 0.5 ng of Ntcp-cRNA or oatp-cRNA in the absence (control) or presence of 5 ng of the indicated oligonucleotides. After 1 day (Ntcp) or 3 days (oatp) of culture, the uptake of 10 μM taurocholate was measured at 25 °C in a medium containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris adjusted to pH 7.5. Uptake values represent means ± S.E.M. for 12–15 determinations in one out of three oocyte preparations. * $P < 0.01$ compared with control.

Ntcp-cRNA injected		oatp-cRNA injected	
Oligonucleotide	Taurocholate uptake (pmol/h per oocyte)	Oligonucleotide	Taurocholate uptake (fmol/h per oocyte)
Uninjected	0.03 ± 0.004*	Uninjected	9.5 ± 0.5*
Control	3.7 ± 0.5	Control	329.2 ± 39.3
Ntcp-sense	3.7 ± 0.6	oatp-sense	398.5 ± 34.2
Ntcp-antisense1	0.03 ± 0.001*	oatp-antisense1	15.0 ± 2.4*
Ntcp-antisense2	0.03 ± 0.005*	oatp-antisense2	14.7 ± 1.3*
oatp-antisense2	3.6 ± 0.7	Ntcp-antisense2	362.3 ± 37.2

Table 2 Effects of sense and antisense oligonucleotides of Ntcp and oatp on Na^+-dependent taurocholate uptake in oocytes injected with total rat liver mRNA

Oocytes were injected with 50 ng of total rat liver mRNA in the presence of 1.25 ng of Ntcp-sense, 2.5 ng of Ntcp-antisense2 or 1.25 ng of oatp-antisense2 oligonucleotides. After 5 days of culture, the uptake of 10 μM taurocholate was measured at 25 °C in a medium containing either 100 mM NaCl or 100 mM choline chloride in 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris adjusted to pH 7.5. Uptake values represent means ± S.E.M. for three independent experiments. * $P < 0.05$, compared with Ntcp-sense and oatp-antisense2; ** $P < 0.05$, compared with Ntcp-sense and Ntcp-antisense2.

	Taurocholate uptake (fmol/h per oocyte)		
Oligonucleotide	NaCl	CholineCl	Net Na^+-dependent uptake
Ntcp-sense	425 ± 91	72 ± 13	354 ± 92
Ntcp-antisense2	115 ± 19*	104 ± 9	11 ± 21*
oatp-antisense2	322 ± 117	35 ± 10**	288 ± 117

oatp-mediated taurocholate uptake by the corresponding antisense oligonucleotides. Maximal specific inhibitory effects were obtained with 2.5 ng of Ntcp-antisense2 and with 1.25 ng of oatp-antisense2 respectively (results not shown). All further experiments were therefore performed with antisense2 oligonucleotides against Ntcp and oatp respectively.

Effect of Ntcp-antisense2 oligonucleotide on the expression of Na^+-dependent taurocholate uptake in *Xenopus laevis* oocytes injected with total rat liver mRNA

As shown in Table 2, co-injection of the Ntcp-sense oligonucleotide together with total rat liver mRNA did not prevent the expression of Na^+-dependent taurocholate uptake in *Xenopus laevis* oocytes. However, the specific Ntcp-antisense2 oligonucleotide inhibited the expression of net Na^+-dependent taurocholate uptake down to 3.2 ± 5.9 % (mean ± S.E.M.) when co-injected with total rat liver mRNA. As a control, co-injection of oatp-antisense2 did not inhibit the expression of net Na^+-dependent taurocholate uptake significantly, although it inhibited the expression of Na^+-independent taurocholate uptake (see below).

Table 3 Effects of sense and antisense oligonucleotides of oatp and Ntcp on Na^+-independent taurocholate and BSP uptake in oocytes injected with total rat liver mRNA

Oocytes were either not injected or injected with 50 ng of total rat liver mRNA in the presence of 1.25 ng of oatp-sense, 1.25 ng of oatp-antisense2 or 2.5 ng of Ntcp-antisense2 oligonucleotides. After 5 days of culture, the uptake of 10 μM taurocholate or 2 μM BSP was measured at 25 °C in a medium containing 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes/Tris adjusted to pH 7.5. Uptake values represent means ± S.E.M. for 20–50 determinations in five (taurocholate) or two (BSP) independent oocyte preparation.; * $P < 0.05$, compared with oatp-sense.

Oligonucleotide	Uptake (fmol/h per oocyte)	
	Taurocholate	BSP
Uninjected	17.7 ± 1.5*	69 ± 4*
oatp-sense	75.6 ± 21.4	597 ± 29
oatp-antisense2	29.6 ± 6.3*	339 ± 23*
Ntcp-antisense2	57.1 ± 11.8	592 ± 26

Effects of oatp-antisense2 oligonucleotide on the expression of Na^+-independent taurocholate and BSP uptake in *Xenopus laevis* oocytes injected with total rat liver mRNA

As demonstrated in Table 3, co-injection of oatp-sense or Ntcp-antisense2 oligonucleotides together with total rat liver mRNA exerted no effects on the expression of either Na^+-independent taurocholate or BSP uptake. In contrast, co-injection of the oatp-antisense2 oligonucleotide together with total rat liver mRNA decreased the expression of Na^+-independent taurocholate uptake to 20.6 ± 13.6 %, but Na^+-independent BSP uptake only down to 51.1 ± 5.3 %. Increasing the concentrations of oatp-antisense2 had no further inhibitory effects on the expression of BSP uptake (results not shown).

DISCUSSION

Previous inhibition and competition studies performed with isolated rat hepatocytes and isolated basolateral membrane vesicles suggested the presence of different uptake systems for bile acids and organic anions in the basolateral membrane of hepatocytes (for review see [26]). Owing to the lack of specific inhibitory antibodies it was almost impossible to study single carriers in these experimental systems. Thus the cloning of Ntcp and oatp made tools available to study these single carriers *in vivo* in rat liver as well as in heterologous expression systems such as *Xenopus laevis* oocytes or eukaryotic cells. In addition, specific antisense oligonucleotides against these cloned carriers became available that should allow selective prevention of their expression and thus permit the study of their importance for bile acid transport within rat hepatocytes. Because primary cultured rat hepatocytes rapidly down-regulate the expression of Ntcp [27] we decided to use *Xenopus laevis* oocytes to study their expression. *Xenopus laevis* oocytes are the only known expression system that permits the injection of unfractionated total mRNA and measurement of its functional expression. Numerous proteins of different organs have been cloned with this expression system [28]. Thus expression of receptors, channels and secreted proteins as well as plasma membrane and mitochondrial transporters [28,29] at their correct cellular locations indicate that eukaryotic mRNAs are efficiently and quantitatively expressed in *Xenopus laevis* oocytes. This assumption was further supported by the linear correlation between expression of Ntcp and oatp with the amounts of cRNA injected (results not shown). As has been demonstrated for receptors [30], channels [31,32] and co-transporters [33,34] we wished to block the expression of Ntcp and oatp in oocytes by antisense oligonucleotides and investigate their effects on bile acid and BSP transport.

The present study demonstrates that injection of specific antisense oligonucleotides against cloned Ntcp and oatp blocked almost completely the expression of taurocholate uptake activity by total rat liver mRNA. These results indicate that Ntcp and oatp could account for most, if not all, bile acid uptake activities in rat liver. Obviously this conclusion is based on the assumption that Ntcp and oatp represent the only basolateral bile acid uptake systems in hepatocytes. Although this assumption could only be proved in functionally intact (i.e. differentiated) isolated hepatocytes, it is noteworthy that in both hepatocytes and mRNA-injected oocytes the relative proportion of maximal Na^+-independent to maximal Na^+-dependent taurocholate uptake rates was found to be approx. 20 % [24,35]. A similar 5:1 relationship between Na^+-dependent and Na^+-independent taurocholate uptake was found in this study (Tables 2 and 3). These similarities between relative expression levels of Na^+-dependent and Na^+-independent taurocholate uptake activities in hepatocytes and mRNA-injected oocytes further indicate that the observed specific inhibitory patterns by the Ntcp and oatp antisense oligonucleotides (Tables 2 and 3) can be quantitatively related to liver cell function, and that no specific messages encoding other bile acid uptake proteins have been lost during the process of mRNA isolation and expression.

In contrast with bile acids, Na^+-independent BSP uptake could only be inhibited by approx. 50 % by the oatp-antisense2 oligonucleotide (Table 3). Hence it is most probable that additional hepatocellular uptake systems for amphipathic organic anions are present in rat liver. These could include previously proposed candidate proteins such as the BSP/bilirubin binding protein [14], a 55 kDa organic anion binding protein [4] or the 37 kDa ‘bilitranslocase’ [9]. However, the exact transport functions of these candidate BSP carrier proteins remain to be determined. Because oatp extends its substrate specificity to ouabain, endogenous steroids and n-pentylajmalinium [21], the assumed additional organic anion transporters might exhibit a more specific substrate requirement for organic anions.

In conclusion, our studies indicate that in *Xenopus laevis* oocytes injected with rat liver mRNA the cloned Ntcp represents the major, if not the only, Na^+-dependent taurocholate uptake system. In addition, oatp is responsible for most of Na^+-independent taurocholate transport but for only approximately half of BSP uptake, suggesting the presence of additional organic anion transporters in rat liver. The cloning of such additional carriers is currently being attempted in our laboratory.

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