J. Membrane Biol. 160, 1–8 (1997)

The Journal of
Membrane
Biology

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Topical Review

Molecular Properties of Hepatic Uptake Systems for Bile Acids and Organic Anions

B. Hagenbuch
Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, CH-8091 Zurich, Switzerland

Received: 13 March 1997/Revised: 3 June 1997

Introduction

Transport proteins are a necessity for the proper function of the liver and are intrinsically involved in all of the major functions of hepatocytes such as, for example, bile formation, detoxification and metabolism. Bile provides a main excretory pathway for various organic compounds that cannot be eliminated by the kidneys. Therefore, to generate ongoing bile flow hepatocytes must continuously transport bile acids from portal blood into bile. This transcellular transport process is driven by several active transport proteins at the basolateral and the canalicular membrane [53]. To facilitate the elimination of endogenous and exogenous xenobiotics, including drugs and their metabolites, the liver contains a lot of enzyme systems that convert lipophilic compounds to more polar derivatives by oxidation and reduction, or perform conjugation reactions to allow quick excretion either into urine or into bile [64, 138]. To extract these diverse xenobiotics efficiently from portal blood and to make them available to the converting enzymes, hepatocytes possess many distinct transport systems in the basolateral membrane [99]. After conjugation, the newly formed compounds have to be transported by specific carriers out of hepatocytes back into blood for renal secretion [17] or they have to be excreted by distinct carriers at the canalicular membrane into bile [95]. In addition, for proper metabolism, transport proteins for metabolic substrates such as, for example, glucose [119], amino acids [92], Krebs cycle intermediates [140] and coenzymes [61] are also found in the hepatocyte plasma membrane.

Functional Characterization of Transport Systems in Hepatocytes

Initially, elimination of compounds by the liver was studied using the isolated perfused rat liver. With this experimental setup general conditions as, for example, the dependency on sodium ions for the transport of bile acids could be determined [25, 103]. With the possibility to isolate and culture primary hepatocytes, studies were undertaken to examine the mechanisms and substrate specificity of bile acid transport [6, 124]. After establishing techniques to isolate membrane vesicles from the two functionally different hepatocyte surface domains, the sinusoidal and the canalicular membrane, respectively [13, 90], transport of various substrates could be studied under well defined experimental conditions. Using all these experimental systems various transport systems for compounds that are taken up into hepatocytes have been characterized and their transport characteristics have been determined (Table 1). Moreover, most of these functionally characterized transport systems have their functional counterparts in other epithelial tissues such as the small intestine, the kidney or the choroid plexus [101]. Quantitatively, the bile acids are the major cholephilic organic anions. Their uptake into hepatocytes has been extensively studied and shown to be mediated by both Na^+-dependent as well as Na^+-independent transport systems [53].

Correspondence to: B. Hagenbuch

Abbreviations: Ntcp1, Na^+/taurocholate cotransporting polypeptide; mEH, microsomal epoxide hydrolase; BSP, bromosulfophthalein; oatp1, organic anion transporting polypeptide

Key words: Bile acids — Liver transport — Sodium-taurocholate cotransport — Organic anion transport

Table 1. Transport systems in the plasma membrane of hepatocytes

Transport systems for metabolic substrates	Functionally characterized	Cloned or expressed[1]
Glucose transporters		
Facilitated glucose transport	[31]	[39, 70, 94, 120]
Na^+-dependent glucose transport		[76, 86]
Amino acid transporters		
System A	[87]	
System ASC	[73]	[8, 109]
System X_{AG}^-	[44]	[68]
System x_c^-	[88, 118]	
System N	[72]	
System n	[32, 97, 105]	
System y^+	[130]	[24, 74, 129]
System GLY	[22]	[114]
System L	[112]	[11]
System β	[20, 59]	[123]
Peptide transporters		
Glutathione transport	[96]	[136, 137]
Oligopeptide transport	[139]	[33, 84]
Mono- and dicarboxylates		
Fatty acid transport	[116]	
H^+-coupled pyruvate and lactate transport	[29]	[42]
Na^+/α-ketoglutarate cotransport	[140]	[98]
Cholate/α-ketoglutarate exchanger	[16]	
Nucleosides		
Na^+/adenosine cotransport	[93]	[21]
Na^+/uridine cotransport, Na^+-independent uridine transport	[91]	
Transport systems for cholephilic compounds and drugs		
Na^+/bile acid cotransport	[108]	[55]
Na^+-independent bile acid transport	[6]	[63]
Prostaglandin transport	[2]	[58, 67]
Metothrexate transport	[61]	[106]
Organic cation transport	[15]	[47]
Leukotriene transport	[81]	

[1] Cloned or expressed means either cloned from liver or cloned from some other tissue but expressed in the liver based on a positive Northern blot.

Na^+-dependent Bile Acid Uptake Systems

Using different experimental setups it has been shown that the Na^+-dependent bile acid uptake system preferentially transports taurocholate and other conjugated bile acids [7, 16, 124, 135]. But on the basis of inhibition kinetics a broader substrate specificity was proposed [141]. K_m values between 17 and 33 μM for taurocholate have been demonstrated [19, 141] and transport has been shown to be electrogenic [83]. Na^+-dependent bile acid transport was found to be selectively expressed in differentiated mammalian hepatocytes [36, 37, 128] and was first detected at 20 days of gestation in rat liver [117]. It is downregulated after biliary diversion [1, 60] and upregulated during fasting and in the postpartum period [27, 40]. In addition, stimulation of Na^+-dependent taurocholate transport by cyclic AMP has been documented [48]. To find out whether all these properties could be attributed to a single transport protein or whether several Na^+-dependent bile acid transport systems with a narrow but overlapping substrate specificity and slightly different affinities for taurocholate would be present in the hepatocyte plasma membrane, it was necessary to clone this transport function and to determine its molecular properties. Using expression cloning in *Xenopus laevis* oocytes a Na^+/taurocholate cotransporting polypeptide (Ntcp1) was cloned and characterized [55] and shown to be able to account for essentially all the characteristics of Na^+-dependent taurocholate uptake in normal and diseased liver [53]. In addition, using photoaffinity labeling [128] and monoclonal antibodies [4], a protein involved in Na^+-dependent bile acid transport was identified which later has been shown to be identical to microsomal epoxide hydrolase [126]. More recently, Madin-Darby canine kidney cells stably transfected with the cDNA for microsomal epoxide hydrolase (mEH) have been shown to mediate Na^+-dependent uptake of taurocholate and to an even higher extent Na^+-dependent uptake of cholate [127]. From these data it was concluded that Ntcp1 would primarily transport taurocholate while mEH would predominantly mediate Na^+-dependent uptake of cholate.

Na^+-independent Bile Acid Uptake Systems

The Na^+-independent bile acid uptake system is the ontogenically older transport mechanism, since in lower vertebrates such as the little skate or the rainbow trout only Na^+-independent transport of bile acids could be demonstrated [37, 102]. In mammalian hepatocytes, mainly unconjugated bile acids such as cholate are transported in a Na^+-independent way [38, 99, 124]. Several exchange mechanisms for this carrier-mediated uptake have been proposed. Cholate could be exchanged for OH^- [14, 125], for sulfate [62] or for dicarboxylates [16, 140]. In addition and most interestingly, based on kinetic inhibition experiments, the Na^+-independent bile acid uptake system has been proposed to be a ''multispecific bile salt transporter'' which could transport besides unconjugated and conjugated bile acids a broad range of substrates such as bromosulfophthalein (BSP), cardiac glycosides, neutral steroids, peptides and various drugs [38, 99]. Again, to assign a certain transport function or

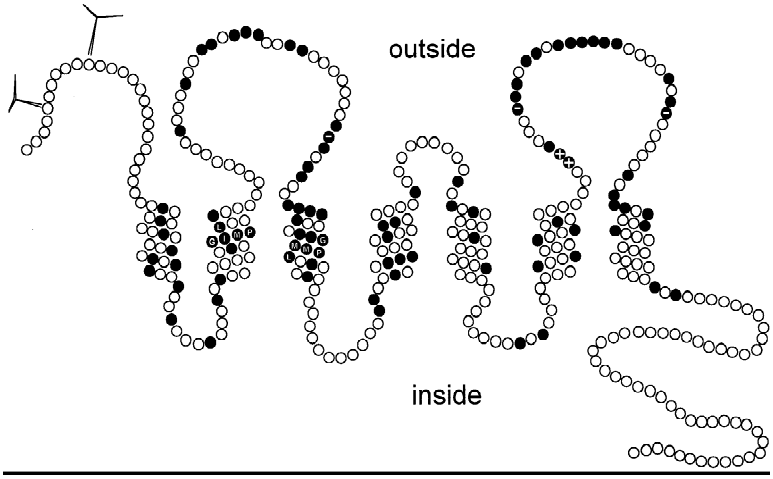

Fig. 1. Proposed model of Ntcp1
N-glycosylation sites at positions five and eleven of Ntcp1 are marked. Black circles represent identical amino acid residues in all eight Na^+/bile acid cotransporters (*see* Table 2). The conserved repeated motif in transmembrane domains two and three as well as the conserved charged amino acid residues are indicated.

a broad substrate specificity to a distinct transport protein, the cloning and determination of the molecular properties of this transport systems was required. Expression cloning in *Xenopus laevis* oocytes was again used to isolate an organic anion transporting polypeptide (oatp1) [63] which after functional expression in stably transfected cells and in *Xenopus laevis* oocytes has been shown to be able to transport besides BSP and bile acids [63, 79] a wide range of compounds (*see below*).

In the following, I will summarize the molecular properties of the cloned bile acid and organic anion transport systems, the Na^+/taurocholate cotransporting polypeptide (Ntcp1) [55] and the organic anion transporting polypeptide (oatp1) [63] and compare them with their related transport proteins so far cloned from liver, small intestine and kidney of different species.

The Na^+/Taurocholate Cotransporting Polypeptide (Ntcp1)

The classical biochemical approach based on photoaffinity and chemical labeling, immunological techniques and solubilization/reconstitution proved difficult to unequivocally identify and isolate the basolateral Na^+-dependent bile acid uptake system [49]. With the help of techniques from molecular biology, Na^+-dependent bile acid uptake was functionally expressed [50] and the Na^+/taurocholate cotransporting polypeptide (Ntcp1) was cloned using the *Xenopus laevis* expression system [55]. Using antisense experiments in *Xenopus laevis* oocytes expression of Na^+-dependent taurocholate transport was almost completely blocked by Ntcp1-specific antisense oligonucleotides while control oligonucleotides had no effect [54]. These results suggest that Ntcp1 represents the major Na^+-dependent taurocholate uptake system in rat liver. In addition, the cloned Ntcp1 was functionally characterized in different mammalian expression systems including COS-7 [19], CHO [107], hepatocyte-like HPCT-1E3 [100] and rat hepatoma cells [122], and shown to account for most characteristics of the previously functionally characterized Na^+-dependent bile acid transport system [89]. Ntcp1 preferentially transports conjugated bile acids [19, 56, 107] and has a K_m for taurocholate between 11 and 30 μM depending on the expression system used [19, 55, 122]. It mediates Na^+/taurocholate cotransport electrogenically with a sodium to taurocholate stoichiometry of 2:1 [53]. Ntcp1 is only expressed in differentiated mammalian hepatocytes [18]. It is downregulated in primary cultures of rat hepatocytes [82] during hepatic regeneration [46] and during different forms of cholestasis [43, 45, 113] and upregulated by prolactin [41]. In addition, the rat *Ntcp*-gene which consists of 5 exons and spans approximately 16 kb of genomic sequence has been isolated and its promoter has been partially characterized [69].

The protein with a native apparent molecular mass of 51 kDa [3, 115] consists of 362 amino acids (Fig. 1). Although there are five potential N-glycosylation sites [55], site-directed mutagenesis has revealed that the protein is only N-glycosylated at positions 5 and 11. This places the amino terminus of the protein to the extracellular side of the plasma membrane (Fig. 1). Antibodies against fusion proteins containing the carboxy terminal end of Ntcp1 were only able to react with the native protein if hepatocytes were permeabilized with detergent [3, 115]. Consequently, the COOH terminus of Ntcp1 has to be located on the cytoplasmic side of the membrane. Depending on the algorithm used, hydropathy analysis of the predicted protein yielded 6 [80], 7 [75] or 8 [30] transmembrane domains. Since only the seven transmembrane domain topology was consistent with the amino terminal end on the extracellular side and the carboxy terminal end on the cytoplasmic side of the plasma membrane (Fig. 1), it was concluded that the rat Ntcp1 is a seven transmembrane domain glycoprotein with struc-

Table 2. The Na^+/bile acid cotransporter family

	Abbreviation	Cloned from	Amino acids	Identity to Ntcp1 (%)	Similarity to Ntcp1 (%)	Reference
Na^+/taurocholate cotransporting polypeptide	Ntcp1	Rat liver	362	100	100	[55]
	Ntcp1	Mouse liver	362	91.4	95.3	[52]
	Ntcp2	Mouse liver	317	90.5	95.3	[52]
	NTCP1	Human liver	349	77.4	88.8	[51]
Ileal sodium bile acid cotransporter	ISBT	Hamster ileum	348	35.8	63.3	[133]
	HISBT	Human ileum	348	34.6	59.2	[134]
	ASBT	Rat ileum	348	36.8	63.8	[23, 111]
		Rabbit ileum	348	37.3	62.7	(1)
P3 proteins of unknown function	P3	Human placenta	477	31.7	54.8	[2]
	P3	Mouse genomic DNA	182 (fragment)	25.4	47.5	[34]

(1) S. Stengelin, S. Apel, W. Becker, M. Maier, J. Rosenberger, G. Wess, W. Kramer. Unpublished sequence submitted to Genbank with the accession number Q28727.

tural similarities to the protein superfamily of rhodopsin [71] and the G protein-linked receptors [35]. Additional members of the sodium/bile acid symporter family [104] have been cloned and their amino acid sequences have been determined (Table 2). The different Na^+/bile acid cotransporters consist of 348 to 362 amino acids with the exception of a short Ntcp2 in the mouse which is missing the last 45 amino acids encoded by exon 5 [52]. The homologies to the rat Ntcp1 are highest among the liver transporters but amount to 35–37% amino acid identity for the ileal proteins. The P3 proteins cloned from human [2] and mouse [34] have also been assigned to be members of this family [104] but are omitted from the alignment discussed in the following section because of their unknown function.

If the amino acid sequences of the eight Na^+/bile acid cotransporters are aligned, 95 amino acids out of 362 are identical among all eight proteins (Fig. 1). It is interesting to note that the amino terminal end of the eight proteins are N-glycosylated but otherwise not conserved. Thus this glycosylation might be important for the proper functioning of the cotransporters or the proper targeting to the plasma membrane [57]. Similarly, at the carboxy terminal end of the Na^+/bile acid cotransporters there are no conserved amino acids. This suggests that these amino acids are not important for Na^+-dependent bile acid cotransport and is supported by the finding that the short mouse liver Ntcp2 which is missing the last 45 amino acids nevertheless is able to transport bile acids in a sodium dependent way when expressed in *Xenopus laevis* oocytes [52]. There is a relatively high homology within the first four transmembrane domains. Besides just hydrophobic amino acids there is a repeated motif G(I/M)MPL present in the second and the third transmembrane domain (Fig. 1). So far it is not known whether this motif or the high homology within the first four transmembrane domains are important for transport. Another intriguing finding is the very conserved extracellular loop connecting transmembrane domains six and seven (Fig. 1). This loop has an amino acid identity of 50% among all eight Na^+/bile acid cotransporters with two negative as well as two positive charges conserved among all eight proteins. Whether these conserved charged amino acids are involved in the binding and transport of the positively charged sodium ion and the negatively charged bile salts is not known so far. But site-directed mutagenesis experiments will eventually lead to the identification of essential amino acids and hopefully help to resolve the mechanism of Na^+/bile acid cotransport.

The Organic Anion Transporting Polypeptide (Oatp1)

Photoaffinity and chemical labeling studies have indicated that a 54 kDa protein might be an essential component of the proposed ''multispecific bile salt transporter'' [38]. Besides this, a protein of 55 kDa [10, 131], the 37 kDa ''bilitranslocase'' [121] and a chloride-dependent high affinity BSP uptake system [132] have been suggested as candidate proteins for Na^+-independent transport of BSP. But none of the putative Na^+-independent organic anion carriers had been completely characterized on the molecular level. Using chloride-dependent BSP uptake as the functional assay, an organic anion transporting polypeptide (oatp1) was cloned with the help of *Xenopus laevis* oocytes [63]. This cloned oatp1 was functionally characterized in *Xenopus laevis* oocytes [17, 79] and in transfected HeLa cells [65, 66, 110]. It was shown that oatp1 besides BSP could also mediate Na^+-independent transport of bile acids, anionic steroid conjugates such as estrone-3-sulfate and estradiol-17β-glucuronide, neutral steroids such as ouabain, aldosterone and cortisol and even the amphipathic organic cation APD-ajmalinium [17]. In addition,

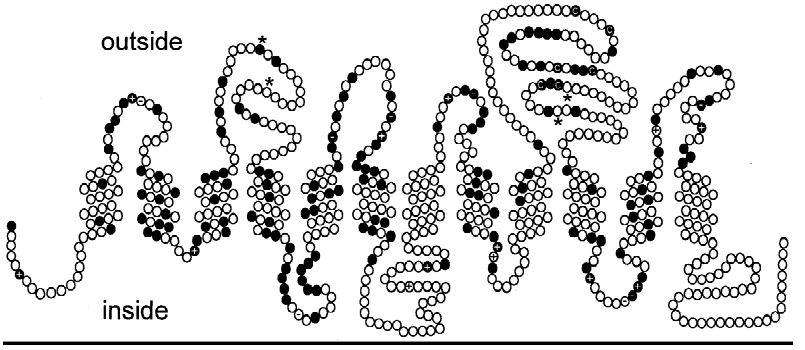

Fig. 2. Proposed model of oatp1
Conserved N-glycosylation sites (*see text*) are shown with an asterisks. Black circles represent identical amino acid residues in all five oatp-related transporters (*see* Table 3). The conserved cystein rich region as well as the conserved charged amino acid residues are indicated.

Table 3. Oatp related transport proteins

	Abbreviation	Cloned from	Amino acids	Identity to oatp1 (%)	Similarity to oatp1 (%)	Reference
Organic anion transporting polypeptide	Oatp1	Rat liver	670	100	100	[63]
	OATP1	Human liver	670	67.0	81.9	[78]
Organic anion transporter	OAT-K1	Rat kidney	669	72.3	83.6	[106]
Prostaglandin transporter	PGT	Rat liver	643	36.3	60.5	[67]
	hPGT	Human kidney	643	37.4	61.1	[85]

it was demonstrated that oatp1 could transport a peptide-based thrombin inhibitor CRC 220 [28] as well as a mycotoxin, ochratoxin A [77]. Thus, oatp1 might indeed represent the previously proposed ''multispecific bile acid transporter'' which besides bile acids would be able to transport a wide range of endogenous as well as exogenous xenobiotics [38, 99].

The glycoprotein with 670 amino acids (Fig. 2) is expressed in the liver basolateral membrane with an apparent molecular mass of 80 kDa [9]. Northern blots suggested a broad tissue distribution [63] and indeed, oatp1 or related proteins have been immunologically detected in the S3 segment of the proximal tubule [9] and in the choroid plexus [5]. While Ntcp1 mRNA could first be detected on day 20 of gestation in rat liver, oatp1 was already expressed on day 16 [18, 26]. This earlier expression during development parallels functional findings in lower vertebrates which show that there are only Na^+-independent transport mechanisms expressed in these phylogenetically older species.

The secondary structure model for oatp1 was originally predicted to contain 10 transmembrane domains [63]. With the cloning and characterization of additional oatp-related proteins (Table 3), a model with 12 transmembrane domains was adopted as a working hypothesis (Fig. 2). In this model, the amino as well as the carboxy terminal end of the protein are located on the cytoplasmic side of the membrane. If all amino acid sequences of the five so far cloned oatp related transporters (Table 3) are aligned, regions of higher and lower homology can be found (Fig. 2). The lowest homology is found at the carboxy terminal end because the prostaglandin transporters are 26 or 27 amino acids shorter than the other three proteins. Thus, as for Ntcp1 it can be speculated that this region is not essential for proper transport function for oatp1. Additional regions with very low homologies are the amino terminal end, transmembrane domains one, seven and twelve and the third intracellular loop. Highly conserved regions among all five transporters are the extracellular loop three (43% identity), the cytoplasmic loop two (57%) and five (38%) and the transmembrane domains two (46%), three (38%), and six (42%). These sequences seem to be the most likely candidates for deletion or replacement experiments in order to establish the transport mechanism of oatp1 and its related proteins. Two conserved features deserve special attention. First, the proposed N-linked glycosylation sites are found in all five proteins in extracellular loops two and five. The first site is identical in all five proteins, the other three are at the exactly same position in at least three of the five transporters (Fig. 2). This might suggest as discussed also for Ntcp1 that N-linked glycosylation is important for either the correct targeting of the transport proteins to the plasma membrane or for its correct functioning. Second, the originally proposed zinc finger domain described for the rat matrin F/G [58], is absolutely retained in all five transport proteins (Fig. 2). This is the first demonstration of such a sequence in transport proteins. It seems highly unlikely that this conservation of such a sequence pattern involving six cystein residues is conserved just accidentally between proteins that share a similar function but rather low amino

acid identity (only 36 to 37% identity of the prostaglandin transporters to oatp1). Therefore, it can be speculated that these cystein residues have some significance in the substrate binding or in the stabilization of the tertiary structure of these carriers. To analyze the transport mechanisms for oatp1 and related proteins and to identify important loops or conserved charged residues for substrate binding and translocation experimental data needs to be obtained. The construction of chimeras, for example, between the closely related liver oatp1 and the renal OAT-K1, which do not transport each others substrates, the deletion of loops or transmembrane domains and finally site directed mutagenesis will eventually support or disprove the above speculations.

Conclusions

The cloning of Ntcp1 and oatp1 and their expression in systems missing intrinsic bile acid transport functions has allowed us to study the substrate specificity of these bile acid carriers. The previously proposed multispecificity could be demonstrated for both transporters although oatp1 accepts a much broader range of compounds than Ntcp1. Future experiments, for example, specific knockout mice for certain transporters will show whether such a multispecific transport protein under physiological conditions indeed transports a broad range of substrates as determined with recombinant proteins or whether the physiological conditions such as, for example, available substrate concentration rather favors one protein one substrate. In addition, the cloning and characterization of additional members of both transport families from additional tissues together with the characterization of these proteins on the genomic level will allow us to determine what regulates the different tissue specific expression as, for example, seen for oatp1 with respect to development in liver and kidney. And finally, the availability of cDNA clones for related transport proteins together with techniques to mutate them or construct chimeric proteins will hopefully lead to the elucidation of the transport mechanisms not only for the bile acid transporters but perhaps even contribute to the better understanding of other Na^+-dependent as well as Na^+-independent transport mechanisms.

This study was supported by the Swiss National Science Foundation Grant 31-45677.95 and the Cloëtta Foundation in Zürich, Switzerland

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