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Generation of a Novel Antibody Probe to the Apical Sodium-Dependent Bile Acid Transporter That Inhibits Ileal Bile Acid Absorption

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Abstract: Intestinal bile acid absorption is mediated by a sodium-dependent transporter located in the brush border apical membrane of ileocytes. The transmembrane topology and the role of individual amino acid residues in the bile acid transport process have been investigated by means of various experimental approaches, leading to multiple hypotheses. We raised a monoclonal antibody against a segment of the transporter comprising vicinal cysteine residues, in order to evaluate its functional role. A 14 amino acid peptide, corresponding to amino acids 104−117 of the transporter, was synthesized, and a monoclonal anti-peptide antibody was raised. In vitro uptake−inhibition studies in the presence of the monoclonal anti-peptide antibody were performed using ileal brush border membrane vesicles. Rabbit ileum was perfused in vivo with 5 mM taurocholic acid in the presence of the monoclonal antibody, and bile acid absorption inhibition was evaluated. The anti-peptide monoclonal antibody significantly reduced the in vitro uptake and in vivo absorption of taurocholic acid. The present data demonstrate the functional relevance of the 104−117 peptide segment and report the generation of a novel antibody against the apical sodium-dependent bile acid transporter (ASBT) that may be used as a therapeutic agent in hypercholesterolemia and in cholestatic pruritus.

Keywords: Apical sodium-dependent bile acid transporter (ASBT); brush border membrane vescicles (BBMV); taurocholic acid (TCA); cholic acid (CA); liver sodium taurocholate transport protein (NTCP)

Introduction

Bile acids are synthesized in the liver and secreted with bile in the small intestine, where they are absorbed and recirculated to the liver.[1,2] In the hepatocytes, bile acids inhibit by a feedback mechanism the activity of cholesterol 7α-hydroxylase, the rate limiting enzyme for their own synthesis from cholesterol.[3] ASBT (apical sodium-dependent bile acid transporter, SLC10A2), localized in the terminal ileum, accounts for most of the active bile acid absorption. The interruption of the enterohepatic circulation of bile acids leads to increased conversion of cholesterol into bile acids in the liver resulting in decreased serum cholesterol levels.[4] Therefore, ASBT is a pharmacological target for hypercholesterolemia.

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Previous photoaffinity labeling studies demonstrated that specific bile acid transport systems are located in the sinusoidal[5] and canalicular[6] membrane of hepatocytes as well as in the brush border[7−9] and basolateral[10] membrane of ileocytes. The ileal sodium dependent bile acid transport also operates in the renal proximal convoluted tubule[11] and in large cholangiocytes.[12,13] These findings have been subsequently confirmed by cloning and molecular characterization of the liver and ileal bile acid transporters,[14] in human and in other mammal species.

Quite recently, cysteine scanning accessibility mutagenesis studies have identified portions of the substrate permeation pathway, protein regions or amino acids involved in recognition, binding and internalization of bile acids and sodium ions.[15−17] In particular, a functional role has been demonstrated for the first extracellular loop (EL1), and it has been suggested that a conserved aspartic acid residue (Asp-122) lining EL1 functions as Na^+ sensor, while Asp-124 reacts with the 7 alpha OH moiety of bile acid steroid ring.[15] Even though the specific role of cysteines at the 105 and 106 positions has not been fully elucidated, previous chemical modification studies of different amino acid side chains with group-specific agents demonstrated the importance of vicinal thiol groups for bile acid uptake into ileal brush border membrane vescicles (BBMV).[9] Therefore, it was proposed that vicinal cysteine residues represent the postulated negatively charged binding site for the sodium ions.[9] ASBT has been cloned and characterized as a 348 amino acid protein with a high interspecies homology,[18−20] and comparison of amino acid sequence in different species reveals that two vicinal cysteine residues at positions 105 and 106 are highly conserved.[21,22] The topological location of the 105 and 106 cysteine residues varies depending on different models of ASBT transmembrane domains.[14] In fact, previous studies suggested either a seven[23] or nine[24] transmembrane domain topology, and a more recent analysis based on dual label

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Amino acid	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
Rabbit ASBT	M	G	C	C	P	G	G	T	A	S	N	I	L	A	Y	W	D
Human ASBT	I	G	C	C	P	G	G	T	A	S	N	I	L	A	Y	W	V
Rat ASBT	M	G	C	C	P	G	G	T	G	S	N	I	L	A	Y	W	I
Mouse ASBT	M	G	C	C	P	G	G	T	G	S	N	I	L	A	Y	W	I
Hamster ASBT	Q	G	C	C	P	G	G	T	A	S	N	I	L	A	Y	W	V

Figure 1. Partial protein sequence alignement of ASBT from different species, showing complete identity of the 104 to 117 amino acid segment (shaded letters) in rabbit, human, and hamster (a single A to G substitution at position 111 is present in rat and mouse ASBT). This segment, containing two vicinal cysteines at positions 105 and 106, was used to raise anti-ASBT monoclonal antibodies (protein sequences available on GenBank).

epitope insertion scanning mutagenesis strongly supports the seven transmembrane model.[25] Furthermore, the 105 and 106 cysteine residues are supposed to be extracellular according to the seven transmembrane models[21,23,25] and not to be located in the vicinity of the bile acid binding region.[21]

Despite recent findings, the exact mechanisms regulating ASBT interaction with sodium ions and bile acids have not been definitely disclosed.[17] Therefore, we followed a different strategy, based on the use of an antibody probe, to investigate the relevance of a specific ASBT segment for the transport activity and to devise a potentially therapeutic antibody functioning as ASBT inhibitor. We raised a monoclonal antibody against a 14 amino acid synthetic peptide corresponding to the amino acids 104−117 of the cloned transporter. The chosen segment is highly conserved in different species and bears the only vicinal cysteine residues of the ASBT amino acid sequence. We tested the antibody probe in transport-inhibition studies both in vitro, using ileal brush border membrane vesicles (BBMVs), and in the in vivo infused rabbit ileum, to verify the suppression of bile acid absorption due to the specific binding of the antibody to its target antigen.

Experimental Section

Bile Acids. Taurocholic acid (TCA) was purchased from Sigma Chemical Co (St. Louis, MO). Radiolabeled cholic acid (CA) [carboxyl-^{14}C]CA (S.A., 40 mCi/mmol) was purchased from Du Pont NEN Products PA) (Boston, MA); radiolabeled TCA [carbonyl-^{14}C]TCA (S.A., 56 mCi/mmol) from Amersham (Amersham, England).

Peptide Synthesis. The peptide sequence corresponding to the amino acids 104−117 of the transport protein was selected because of its complete identity in ASBT from most species[21] and for the presence of two vicinal cysteines at positions 105 and 106 (Figure 1). The peptide was synthesized by the solid phase method using a continuous flow instrument with on line UV monitoring. The stepwise synthesis was carried out by Fmoc/tBu chemistry using a Wang resin derivatized by C-terminal residue. Fmoc-amino acids were coupled in a 4-fold excess using diisopropylcarbodiimide (DIPCDI) in the presence of hydroxybenzotriazole (HOBt). The fluorenylmethoxycarbonyl (Fmoc) group was cleaved with 20% piperidine−DMF solution. Protected peptide was cleaved from the resin by treatment with 88% trifluoroacetic acid, 5% H_2O, 7% triethylsilane. The resulting product was purified by preparative HPLC; purity was 98%. Structure verification was achieved by amino acid analysis and MALDI-TOF mass spectrometry.

Monoclonal Antibodies. The 14 amino acid peptide was conjugated with KLH, and the product was employed for the production of a monoclonal antibody (subclass IgM) by Biosearch Italia S.p.A./ARETA International s.r.l., Gerenzano, Italy. A monoclonal mouse antihuman dendritic reticulum cell antibody (DRC1) was purchased from Dako A/S, Glostrup, Denmark.

Monoclonal Antibody Characterization and Stability. Ileal brush border membrane vesicles (BBMV 25 μg) were prepared from rabbit intestine (see specific section) and solubilized in Laemmli Buffer,[26] separated on 10% SDS−polyacrylamide gel and transferred onto nitrocellulose membrane. Blots were probed with 0.2 μg/mL of the anti-peptide monoclonal antibody or with the negative control (anti-DRC1 antibody), and incubated overnight. Immunodetection was carried out using a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England, U.K.) after incubation with 1:10000 diluted HRP-conjugated goat IgG antimouse immunoglobulins (Cappel, Organon Teknika Corp., West Chester, PA). A chemiluminescent substrate (PowerSignal; Pierce, Rockford, IL) was used for immunodetection, which was performed using a luminograph LB 981 (EG & G Berthold, Bad Wildbad, Germany), using the WinLight software. Stability of the monoclonal antibody in the intestine was tested by probing ileal BBMV blots with the intestinal perfusion media (see specific section below), which were collected before and after the experiment, diluted 1:10000 in the Western blot incubation buffer in order to obtain a 0.2 μg/mL concentration of the anti-peptide monoclonal antibody, and used for overnight incubation of BBMV blots.

Animals. Male New Zealand Rabbits (1800−2200 g b.w.) were used. The animals were allowed the usual commercial

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diet. Food was not withdrawn before the experiments in order to maintain a high blood flow and better hemodynamic conditions.

Transport/Inhibition Studies in Ileal Brush Border Membrane Vesicles (BBMVs). BBMVs were prepared from rabbit jejunum and ileum (New Zealand Rabbits, 2.5−3 kg b.w.) by the Mg^{2+} precipitation method,[27,28] and protein concentration was evaluated by Lowry's method, using bovine serum albumin as a standard.[29] Uptake of radiolabeled TCA in the ileal BBMVs and radiolabeled CA in jejunal BBMVs was determined by the membrane filtration technique.[27,28] This choice was due to the well established specificity of the ileal active sodium coupled bile acid uptake for conjugated bile acids, and the lack of such transport system in the jejunum.[1−11,18]

Brush border membrane vesicles were preincubated with mannitol 300 mM, Tris-Hepes 20 mM (pH 7.4) with 20 μg/mL of anti-peptide monoclonal antibody or 20 μg/mL of antidendritic cell monoclonal antibody as negative control and incubated over 45 min at 25 °C.

The transport in the presence of Na^+ gradient was initiated by adding 30 μL of the ileal vesicle suspension (70−120 μg of proteins) to 110 μL of incubation medium containing 100 mM mannitol, 100 mM NaCl, 20 mM Tris-Hepes (pH 7.4), 50 μM [^{14}C]taurocholate (ileal BBMV). For transport studies in the absence of Na^+ gradient, the incubation media contained 100 mM mannitol, 100 mM KCl, 20 mM Tris-Hepes (pH 7.4), 50 μM [^{14}C]taurocholate. Transport studies of CA in jejunal BBMV were performed following the same procedure, except for the substitution of 50 μM [^{14}C]taurocholate with 50 μM [^{14}C]cholate in the incubation medium.

Infusion Technique for in Vivo Transport Studies. Anesthesia was induced by ketamine and maintained by a gas mixture of O_2, N_2O, Fluothane. Intestine was exposed by median longitudinal laparotomy, and then it was maintained at constant temperature under gauzes soaked in 0.9% NaCl at 37 °C. Distal 20 ± 2 cm of the rabbit ileum was isolated, and an inlet cannula was connected to a syringe and positioned at the proximal extremity of the isolated segment, whose content was flushed by a 5 min perfusion with normal saline at 37 °C, in order to avoid any possible intestinal residual content.

Fifteen milliliters of PBS solution containing 5 mM TCA and either the anti-peptide monoclonal antibodies (200 μg/mL) or control DRC1 monoclonal antibodies (200 μg/mL) was infused and maintained for 20 min in the isolated loop, which had been tied at both ends.

According to a previously described procedure[30] modified for this experimental purpose, immediately before ileal infusion the mesenteric vein draining from the perfused intestine was cannulated by an 18G cannula and the blood was collected at 1 min intervals over 30 min. The blood flow was measured gravimetrically into previously weighed collecting tubes. Six experiments using six different animals were performed both for the anti-ASBT peptide and for the control monoclonal antibody group. Each animal was used for only one single perfusion study. The integrity of the intestinal segment was assessed as already described.[30]

Analytical Methods. Immediately after the experiments, the blood was centrifuged at 5200*g* and the serum was analyzed for the bile acids by the 3-hydroxysteroid dehydrogenase enzymatic assay (Sterognost-3 Pho, Nycomed AS, Torshov, Norway). A possible deconjugation of the bile acid by intestinal bacteria during intestinal infusion was ruled out by the associated evaluation of free cholate by thin layer chromatography, on silica gel G plates (2 mm thickness) (Merck, Darmstadt, FRG).[31]

Statistical Analysis. Statistical significance for differences in TCA absorption in the perfused ileum was evaluated by the Student's *t* test for paired data.

Results

Monoclonal Antibody Characterization and Stability. Immunoblotting of ileal BBMVs using the anti-peptide monoclonal antibody showed protein bands in the range of 45 and 90 kDa (Figure 2), consistent with the glycosylated rabbit ASBT monomer and dimer, respectively.[32] Stability of the antibody in the intestinal infusion medium was also demonstrated, as shown in Figure 2.

Bile Acid Uptake in Brush Border Membrane Vesicles. In the presence of Na^+ gradient ($[Na^+]_{out}$ $[Na^+]_{in}$) the overshoot was observed in the uptake of radiolabeled TCA in ileal vesicles, while no overshoot was observed in the uptake of radiolabeled CA in jejunal vesicles (Figure 3). In the absence of Na^+ no active transport kinetics was found in either segment for the respective radiolabeled compounds (Figure 3). In the jejunal vesicles a rapid increase in [^{14}C]CA content was observed both in the absence and in the presence of Na^+ gradient. In the jejunal vesicles [^{14}C]TCA absorption was actually absent, without reference to the presence of Na^+ ion gradient (data not shown).

In ileal vesicles, [^{14}C]TCA overshoot was abolished in the presence of monoclonal antibodies and overall transport was

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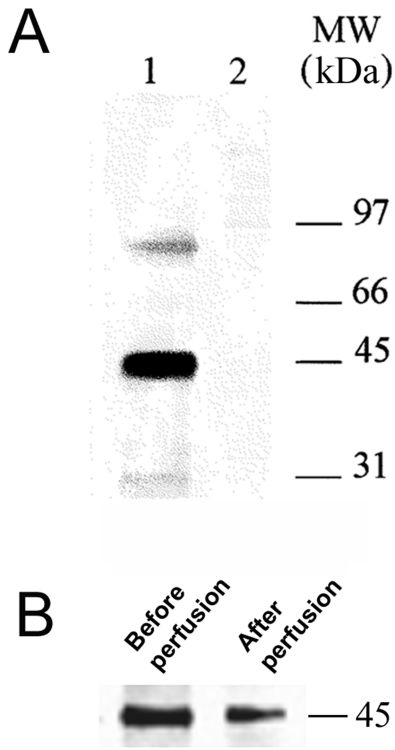

Figure 2. Characterization and stability over time of the monoclonal antibody raised against ASBT. A: Rabbit ileal BBMVs treated as described in the Experimental Section were processed by Western blot. After incubation with the anti-ASBT monoclonal antibody, a protein band was recognized in the range of 45 kDa, consistent with the glycosylated ASBT monomer. A weaker band in the range of 90 kDa was also recognized, consistent with ASBT dimerization. Lane 1, incubation with anti-ASBT monoclonal antibody; lane 2, incubation with anti-DRC1 antibody (negative control). Marker lane is shown on the right. B: Monoclonal antibody stability in the intestinal perfusion buffer. Ileal BBMVs were probed with the diluted intestinal perfusion media collected before and after the perfusion experiment. A band in the range of 45 kDa was observed in both lanes, demonstrating the stability of monoclonal antibody. A weaker signal was observed with the medium collected at the end of the experiment, possibly due to adhesion of the monoclonal antibody to the intestinal wall. Marker lane insert is shown on the right.

reduced by 50% as evaluated by the reduction of the maximal time point determination. On the opposite, no effect was observed in [^{14}C]CA transport in BBMV from jejunum (Figure 3).

In Vivo Perfusion Studies in Rabbit Ileum. After the infusion of the solution with taurocholic acid, serum bile acid levels increased up to a maximum value and then slowly decreased close to the baseline values within ten minutes of the infusion (Figure 4). In the presence of the anti-ASBT peptide monoclonal antibody, the maximal increase in TCA absorption was lower and delayed with respect to the DRC1 monoclonal control antibodies. In particular, the TCA maximal absorption in the presence of the anti-ASBT peptide antibody was 21% of the peak observed in the presence of the DRC1 control antibody ($P < 0.01$).

Discussion

In the present study we have shown the functional inhibition of ASBT in the rabbit ileum by means of a monoclonal antibody raised against a peptide segment of the transporter.

Originally cloned from a hamster intestinal cDNA library,[18] ASBT was subsequently cloned from human,[23] rat,[19] rabbit[32,33] and mouse[34] ileum. ASBT is a glycoprotein consisting of 348 amino acids (347 in the rabbit),[32,33] with a molecular weight in the range of 38−48 kDa in different species, depending on the N-linked glycosylation sites,[6,14,18−21,32] encoded by a major 4.0-kb transcript.[6] The structure−activity relationship and ligand recognition modality have not been completely elucidated. Previous approaches to the study of ASBT topology included the hydropathy analysis according to Kyte−Doolittle and a seven transmembrane model similar to NTCP was proposed, with an extracellular N-terminus and a cytoplasmic C-terminus.[23,35] A nine helix model for human ASBT was successively suggested based on membrane insertion scanning studies,[24] but a more recent N-glycosylation−scanning−mutagenesis approach supported the existence of seven transmembrane domains.[25] So far, it has been documented that the cytoplasmic tail of ASBT plays an important role in its sorting to the apical plasma membrane.[36,37] In particular, ASBT is believed to undergo a transport vesicle-mediated apical sorting pathway, and a 14 amino acid peptide with two phosphorylation residues on the C-terminus seems responsible for its apical targeting.[37]

Before ASBT cloning, it was demonstrated that thiol modifiers inactivate bile acid transport in brush border membrane vesicles, suggesting that two vicinal thiol groups are necessary for sodium dependent transport activity.[9] Site directed mutagenesis studies demonstrated that, among the

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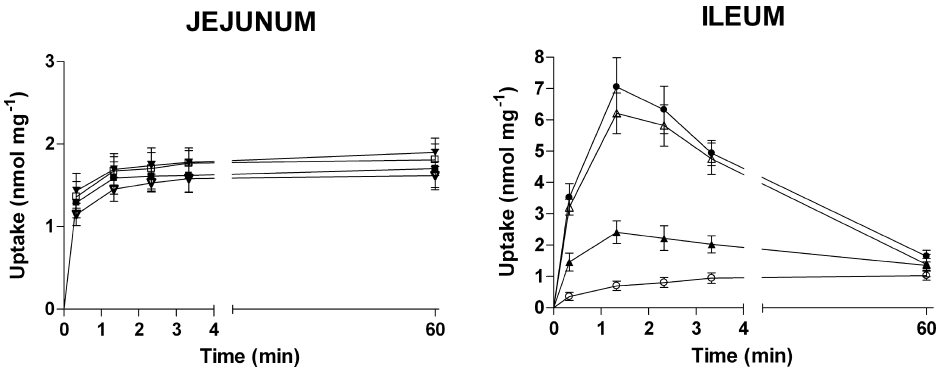

Figure 3. Inhibition of taurocholate transport by monoclonal antibodies against ASBT. Transport of TCA in ileal BBMVs in presence of Na^+ gradient was initiated by adding 30 μL of the vesicle suspension (70−120 μg of proteins) to 110 μL of incubation medium containing 100 mM mannitol, 100 mM NaCl, 20 mM Tris-Hepes (pH 7.4), 50 μM [^{14}C]taurocholate. For transport studies in absence of Na^+ gradient, the incubation media contained 100 mM mannitol, 100 mM KCl, 20 mM Tris-Hepes (pH 7.4), 50 μM [^{14}C]taurocholate. Transport studies of CA in jejunal BBMVs were performed following the same procedure, except for the substitution of 50 μM [^{14}C]taurocholate with 50 μM [^{14}C]cholate in the incubation medium. (Left) Uptake of [^{14}C]cholate in rabbit jejunal BBMVs:(■) [^{14}C]cholate uptake in the presence of Na^+ gradient; (□) [^{14}C]cholate uptake in the absence of Na^+ gradient; (▼) [^{14}C]cholate uptake in the presence of Na^+ gradient plus 20 μg/mL of anti-peptide monoclonal antibody; (▽) [^{14}C]cholate uptake in the presence of Na^+ gradient plus 20 μg/mL of control antibody (DRC1). (Right) Uptake of [^{14}C]taurocholate in rabbit ileal BBMVs: (●) [^{14}C] taurocholate uptake in the presence of Na^+ gradient; (○) [^{14}C]taurocholate uptake in the absence of Na^+ gradient; (▲) [^{14}C]taurocholate uptake in the presence of Na^+ gradient plus 20 μg/mL of anti-peptide monoclonal antibody; (△) [^{14}C]taurocholate uptake in the presence of Na^+ gradient plus 20 μg/mL of control antibody (DRC1). Each point represents the mean ± SD for 6 determinations.

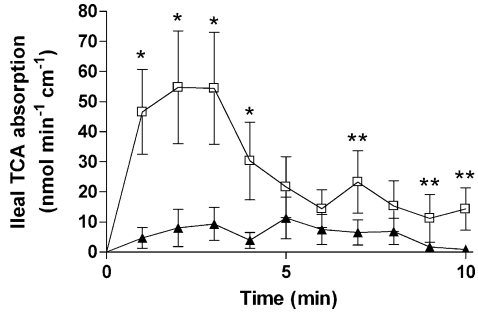

Figure 4. In vivo perfusion studies in rabbit ileum: ileal taurocholate absorption was evaluated after perfusion of the terminal ileum with 5 mM TCA in the presence of anti-ASBT monoclonal antibody. Time point determinations of ileal taurocholate absorption (nmol/min/cm intestine) in the presence of 200 mg/mL anti-ASBT monoclonal antibody (▲) and in the presence of a control monoclonal antibody (DRC1, 200 mg/mL) (□). Each point represents the mean ± SD for 6 determinations. *$P < 0.01$, **$P < 0.05$, Student's t test for paired data.

14 cysteines present in the ASBT sequence, cysteine 270 lies in a sodium sensitive region involved in bile acid transport and that cysteine residues in different positions (namely cysteine residues in the 51, 74, 105, 106, 132, 144, and 225 positions) are also important for transport function.[21] Considering vicinal cysteine groups, Banerjee and co-workers demonstrated that a single substitution of cysteine residues at the 105 and 106 positions with alanine or threonine determines a strong reduction of transport activity,[21] and the inhibition of taurocholate transport was particularly evident for substitution of the cysteine at position 105, which completely abolished the bile acid uptake.[21] Recent findings excluded a direct role of the fourth transmembrane domain (TM4) in sodium ion translocation.[38] Notably, the TM4 region does not include any cysteine residue.[37] Quite recentlly, Hussainzada and co-workers[15] demonstated the functional importance of EL1 for recognition and binding of ASBT substrates. In particular, aspartic acid residues 120 and 122 are supposed to interact with Na^+ and 7 alpha OH moieties, respectively. Further studies from the same group demonstrated that extracellular loop 3 (EL3) is also essential for ASBT activity.[16,17] A model was therefore described, postulating EL1 and EL3 tertiary interaction in the sodium and bile acid binding events and translocation.[15]

We followed a new approach, based on the use of a monoclonal antibody, in oder to confirm the exoplasmic location and the functional relevance of a specific ASBT region, and to test the possibility of interfering with bile acid absorption. Starting from the observations with the thiol modifier agents[9] and with the site directed mutagenesis experiments,[21] we raised and characterized a monoclonal antibody targeted to a short peptide segment, spanning the vicinal cysteine groups at positions 105 and 106 on the first exoplasmic loop of ASBT. A 14 amino acid peptide seemed to be suitable both for a good conjugation with the carrier protein and to raise a well targeted immune response. In fact, a longer peptide could have raised antibodies not strictly

(38) Khantwal, C. M.; Swaan, P. W. Cytosolic half of the transmembrane domain IV of the human bile acid transporter hASBT (SLC10A2) forms part of the substrate translocation pathway. *Biochemistry* **2008**, *47* (12), 3606–3614.

targeted to the segment comprising the two vicinal cysteines. Notably, the complete amino acid sequence of the chosen peptide is completely conserved between rabbit, human and hamster ASBT (Figure 1). Furthermore, we moved from the hypothesis that a peptide segment with high interspecies and interorgan (liver and ileum) identity could bear important functions for the transport process. In fact, seven of the 14 amino acids are conserved also in the liver sinusoidal sodium-dependent bile acid transporter (NTCP),[21] whose overall identity with ASBT is less than 40%.[20]

We observed specific inhibition of bile acid uptake both in vitro (BBMVs) and in vivo (perfused rabbit ileum), indicating that the antibody probe specifically binds to the transporter and impairs its function (Figures 3 and 4). Immunoblotting characterization showed protein bands corresponding to the ASBT monomer and dimer molecular weights (Figure 2). These results support the seven transmembrane model for ASBT, in which the two vicinal cysteines are located on the first extracellular loop. The observed transport inhibition may be due to the blockage of a recognition site for sodium ions or possibly for conjugated bile acids. Alternatively, it could be the result of nonspecific steric hindrance, or perturbation of the conformational changes associated with the substrate recognition and translocation process.

Different authors proposed the negatively charged amino acids D115, E257[39] and E282[40] as potential binding sites for sodium ions at the outer surface of ASBT and NTCP.[41] Our experimental approach, which involves both in vitro and in vivo studies, confirms the previous in vitro findings based on the use of thiol modifiers[9] and amino acid substitution.[21] On the other hand, the different models for ASBT interaction with sodium ions are not necessarily incompatible with each other, since a redundancy of negatively charged amino acids could be necessary for the highly efficient electrogenic transport activity of ASBT.

These results may be helpful in designing new strategies for the inhibition of intestinal bile acid reabsorption both in hypercholesterolemia and in the treatment of cholestatic pruritus.[42] Conversely, they provide a better understanding for the use of ASBT as a drug delivery target.

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