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The novel putative bile acid transporter SLC10A5 is highly expressed in liver and kidney

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Abstract

Here we report the identification, cloning, and characterization of SLC10A5, which is a new member of Solute Carrier Family 10 (SLC10), also known as the "sodium/bile acid cotransporter family". Expression of SLC10A5/Slc10a5 was examined by quantitative real-time PCR and revealed its highest expression levels in liver and kidney in humans, rat and mouse. In rat liver and kidney, Slc10a5 expression was localized by *in situ* hybridization to hepatocytes and proximal tubules, respectively. A SLC10A5-FLAG fusion protein was expressed in HEK293 cells and showed an apparent molecular weight of 42 kDa after immunoprecipitation. When expressed in *Xenopus laevis* oocytes, the SLC10A5-FLAG protein was detected in the oocyte's plasma membrane but showed no transport activity for taurocholate, cholate, estrone-3-sulfate, or dehydroepiandrosterone sulfate. As bile acid carriers are the most related carriers to SLC10A5 though, we strongly suppose that SLC10A5 is an orphan carrier with yet non-identified substrates.
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The enterohepatic circulation of bile acids is an important mechanism to conserve these cholesterol derivatives from the intestine and reduce energy-wasting de novo bile acid synthesis in the liver. Two bile acid carriers of the Solute Carrier Family SLC10 are involved in this process: the apical sodium-dependent bile acid transporter ASBT (SLC10A2) and the hepatic Na^+/taurocholate cotransporting polypeptide NTCP (SLC10A1) [1,2]. ASBT is highly expressed in the apical brush border membrane of enterocytes of the terminal ileum [3], whereas NTCP is exclusively expressed at the basolateral membrane of hepatocytes [4]. Besides the intestine, ASBT expression was also detected in the apical membrane of renal proximal convoluted tubules, where it allows tubular reabsorption of bile acids that are filtered by the glomeruli [5]. Both carriers mediate sodium-coupled uptake of physiological bile acids such as taurocholate. Additionally, NTCP but not ASBT mediates uptake of sulfoconjugated steroid hormones such as estrone-3-sulfate [6,7]. Carriers of the Organic Anion Transporting Polypeptide (SLCO) carrier family that are highly expressed at the basolateral membrane of hepatocytes such as OATP1B1 and OATP1B3 are also involved in liver uptake of bile acids [8,9]. In contrast to NTCP, these OATP carriers show a sodium-independent transport mechanism.

Here we report on the cloning and characterization of a novel carrier of the SLC10 carrier family, SLC10A5, which does not transport bile acids such as taurocholate and cholate. Based on its tissue selective expression in liver and kidney SLC10A5, however, is probably involved in the hepatic and renal transport of other classes of bile acids.

Materials and methods

Materials. The radiochemicals [1,2,6,7-^{3}H(N)]dehydroepiandrosterone sulfate (60 Ci/mmol), [6,7-^{3}H(N)]estrone-3-sulfate (57.3 Ci/mmol), and [^{3}H]taurocholate (3.5 Ci/mmol) were purchased from PerkinElmer Life

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Sciences. [^{14}C]Cholate (55 mCi/mmol) was obtained from American Radiolabeled Chemicals.

Cloning of human, mouse, and rat SLC10A5/Slc10a5 cDNAs. Several hypothetical proteins that all contain the SBF-domain of the *Sodium Bile Acid Symporter Family* (Pfam01758) were predicted from the human genome sequence by automated computational analysis. For one of these sequences, further referred to as SLC10A5, homologous sequences existed also for rat and mouse that are further referred to as Slc10a5. These sequences were used for an RT-PCR-based strategy to obtain the full open reading frame (ORF) of the hypothetical SBF-related transporter proteins. The following oligonucleotide primers were designed for PCR amplification based on the cDNA sequences of GenBank Accession Nos. XM_376781 and XM_143078: 5′-agc tga gct cat gat tag aaa act ttt tat tg-3′ forward and 5′-ttg tga gct cat tgt tag att agg aaa ttt c-3′ reverse for human SLC10A5 and 5′-tca ccc gcg gtt gtt taa act ttc aaa atg tct g-3′ forward and 5′-gat cta gac tgt aca ttt taa act aga gga g-3′ reverse for mouse Slc10a5. RT-PCR was performed from 1 μg human and mouse liver poly(A)$^+$RNA (Clontech) using the Expand High Fidelity PCR system (Roche) according to the following thermocycling conditions: 1 cycle of 94 °C × 2 min; 10 cycles of 94 °C × 15 s, 60 °C × 30 s minus 0.5 °C each cycle, and 72 °C × 1 min; 30 cycles of 94 °C × 15 s, 55 °C × 30 s, and 72 °C × 1 min plus 5 s each cycle; and a final extension of 72 °C × 10 min. We obtained SLC10A5/Slc10a5 amplicons of ~1300 bp which were gel purified and cloned downstream from the T3 promoter into pBluescript (Stratagene). Three clones of each species were sequenced on both strands, and the sequences were deposited into the GenBank database with Accession Nos. AY825924 for human SLC10A5 and AY825925 for mouse Slc10a5. In order to obtain the Slc10a5 orthologue from rat also, we used the same approach as described above using RT-PCR amplification of 1 μg rat small intestine poly(A)$^+$RNA with the following primers: 5′-atg tct gga aaa ctt ttc ata att c-3′ forward and 5′-tta aac gag agg agc ctt ttc-3′ reverse. The rat Slc10a5 PCR amplicon of ~1300 bp was gel purified and ligated into pGEM-T (Promega). Three clones were sequenced on both strands and the sequence was deposited into the GenBank database with Accession No. DQ074435. For *in situ* hybridization experiments, this Slc10a5-pGEM-T construct was used. For expression experiments in *Xenopus laevis* oocytes, the full length rat Slc10a5 cDNA sequence was subcloned into pBluescript as described above.

Real-time quantitative PCR. Using cDNA panels of 12 human tissues, 9 rat tissues, and 18 mouse tissues (Clontech) we performed quantitative expression analyses with ABI PRISM 7300 technology (Applied Biosystems). The PCR amplification was performed with TaqMan Gene Expression Assays Hs01049585 for human SLC10A5, Rn02585862 for rat Slc10a5, and Mm02345249 for mouse Slc10a5. For endogenous control, expression of beta actin was analyzed in each tissue using Expression Assays Hs99999903 for human tissues, Rn00667869 for rat tissues, and Mm00607939 for mouse tissues. For each tissue, quadruplicate determinations were performed in a 96 wells optical plate using 5 μl cDNA, 1.25 μl TaqMan Gene Expression Assay, 12.5 μl of TaqMan Universal PCR Master Mix, and 6.25 μl of water in each 25 μl reaction. Plates were heated for 10 min at 95 °C, and then 45 cycles of 15 s at 95 °C and 60 s at 60 °C were applied. Relative SLC10A5/Slc10a5 expression (ΔC_T) was calculated by subtracting the signal threshold cycle (C_T) of the endogenous control from the C_T value of SLC10A5/Slc10a5. Subsequently, $\Delta\Delta C_T$ values were obtained for each tissue by subtracting the ΔC_T of the tissue with the lowest SLC10A5/Slc10a5 expression from the ΔC_T of each individual tissue.

In situ hybridization (ISH) of rat liver and kidney sections. DIG-labeled rat Slc10a5 cRNA-probes were synthesized from the rat Slc10a5-pGEM-T plasmid as described [10]. Briefly, plasmid DNA was digested with SacII and SacI (New England Biolabs) for the production of the Slc10a5-sense-cRNA and Slc10a5-antisense-cRNA, respectively. Subsequently, *in vitro* transcription was performed using the 10 × RNA-DIG Labeling-Mix (Boehringer Mannheim) by T7-RNA polymerase and SP6-RNA polymerase (Promega). Slc10a5 *in situ* hybridization was performed in rat liver and kidney samples with normal histologies as described [10]. Staining was visualized by developing sections with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate in a humidified chamber protected from light and sections were mounted in Glycergel (Dako).

Cloning of NTCP cDNA. NTCP cDNA was cloned for comparative analysis. Briefly, the full open reading frame of human NTCP was amplified by RT-PCR from 1 μg liver RNA (Clontech). The following gene-specific oligonucleotide primers were used: 5′-gcg gta ccg gat gga ggc cca caa c-3′ forward and 5′-tct ctc gag cta ggc tgt gca ag-3′ reverse. PCR amplification was performed using the Expand High Fidelity PCR system (Roche) as described above. The PCR product of the expected size was gel purified and cloned downstream from the T3 promoter into pBluescript (Stratagene). Sequence verification was done according to the reference sequence with GenBank Accession No. NM_003049.

Transport studies in X. laevis oocytes. The pBluescript plasmids containing the cloned SLC10A5/Slc10a5 and NTCP cDNAs were linearized and used as a template to generate a capped cRNA. *Xenopus laevis* frogs were anesthetized by exposure for 10 min to a 1% solution of 3-aminobenzoic acid ethyl ester (MS-222), and oocytes were prepared as described [11]. After overnight incubation at 18 °C, defolliculated oocytes were selected and microinjected with 4.6 ng (46 nl) SLC10A5 cRNAs or with a corresponding 46 nl volume of water. The oocyte medium was changed daily and healthy oocytes were selected for experiments. After culture for three days in modified Barth's medium at 18 °C, uptake of radiolabeled substrates 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, pH 7.5. After washing with the same buffer, each individual oocyte was dissolved in 500 μl of 10% SDS and 4 ml scintillation fluid, and the radioactivity was counted in a liquid scintillation counter.

Immunofluorescence detection of SLC10A5-FLAG fusion proteins in X. laevis oocytes. The SLC10A5/Slc10a5 cDNAs were elongated by the sequence 5′-gattacaaggatgacgacgataag-3′, coding for the FLAG-epitope (DYKDDDDK), using QuikChange site-directed mutagenesis (Stratagene) and FLAG insertions were verified by DNA sequencing. *Xenopus laevis* oocytes were prepared and injected with the SLC10A5-FLAG cRNAs as described above. Three days after cRNA injection, the vitelline membrane was removed, and the oocytes were fixed in Dent's fixans (80% methanol/20% dimethylsulfoxide). Oocytes were washed in decreasing concentrations of methanol (90%, 70%, 50%, and 30%) in PBS (phosphate buffered saline containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 7.3 mM Na_2HPO_4 at pH 7.4, 37 °C) and were incubated with the monoclonal mouse anti-FLAG antibody (Sigma) at 1:1000 in blocking solution containing 1% BSA and 4% goat serum (Dako Cytomation) in PBS overnight at 4 °C. Oocytes were washed 11 times with PBS and were incubated with the Alexa Fluor 488 labeled goat anti-mouse IgG secondary antibody (Molecular Probes) at 1:500 in blocking solution for 2 h at room temperature. After a second washing step with PBS, oocytes were fixed with 3.7% formaldehyde in PBS and washed with ascending concentrations of ethanol (30%, 50%, 70%, and 100%) in PBS. Oocytes were embedded in Technovit 7100 (Heraeus Kulzer). Finally, 5 μm sections were cut, and the FLAG-tagged SLC10A5 proteins were detected by fluorescence microscopy (Leica DM6000B fluorescence microscope).

Immunoprecipitation of the SLC10A5-FLAG protein. The mouse Slc10a5-FLAG cDNA construct was excised from the pBluescript plasmid and subcloned into the pcDNA5/TO mammalian expression vector (Invitrogen). In the generated Slc10a5-FLAG-pcDNA5 vector, expression of the SLC10A5 protein is under the control of the cytomegalovirus (CMV) promoter and the tetracycline operator sequence (*tetO*$_2$). The Human Embryonic Kidney 293 cell line (HEK293) was maintained in D-MEM/F12 medium (Gibco) supplemented with 10% fetal calf serum (Sigma), L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C, 5% CO_2, and 95% humidity. HEK293 cells were seeded in 6-well plates coated with poly-D-lysine at a density of 1×10^6 cells per well in antibiotic free D-MEM/F12 medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. On the following day, the cells were transfected with 4 μg of the mouse Slc10a5-pcDNA5 vector or with 4 μg pcDNA5/TO vector alone (control) by lipofectamine 2000 reagent and processed for radioimmunoprecipitation as described [12]. The samples were separated by 12% SDS–polyacrylamide gel electrophoresis and exposed to Kodak BioMax MR film (Sigma) at −80 °C.

Results

In 2004 we searched the available nucleotide and genomic databases for novel bile acid carriers using the amino acid and cDNA sequences of NTCP and ASBT as queries. Besides these two carriers, we identified two hitherto uncharacterized sequences (GenBank Accession Nos. XM_376781 from human and XM_143078 from mouse), which were predicted by automated computational analysis from genomic sequences. Using an RT-PCR based approach for cloning of the predicted open reading frames, we obtained 1317 and 1305 bp transcripts from human and mouse livers, respectively. Using a similar approach, we also cloned a 1305 bp transcript from rat small intestine. These sequences were deposited into the GenBank database with Accession Nos. AY825924, AY825925, and DQ074435 and are henceforth referred to as human SLC10A5, mouse Slc10a5, and rat Slc10a5, respectively. The cloned human SLC10A5 cDNA sequence is coding for a 438 amino acid membrane protein and the rat and mouse SLC10A5 proteins consist of 434 amino acids. The SLC10A5 proteins show the highest sequence identity/similarity (34%/61%) to the orphan carrier SLC10A3 and a lower sequence identity/similarity (22%/44%) to the bile acid carriers NTCP and ASBT. Fig. 1 shows an alignment of the deduced amino acid sequences of the human, rat, and mouse SLC10A5 proteins, which exhibit an overall sequence identity of >70%. Based on the analysis of three different topology prediction programs, SLC10A5 shows nine alpha-helical transmembrane domains with an extracellular orientation of the N-terminus and an intracellular location of the C-terminus. One conserved putative *N*-glycosylation site was detected in the SLC10A5 N-terminus (N^{99} in humans, and N^{96} in rat and mouse).

SLC10A5/Slc10a5 mRNA expression was analyzed in different human, rat, and mouse tissues by real-time quantitative PCR using multiple tissue cDNA panels (Fig. 2). In

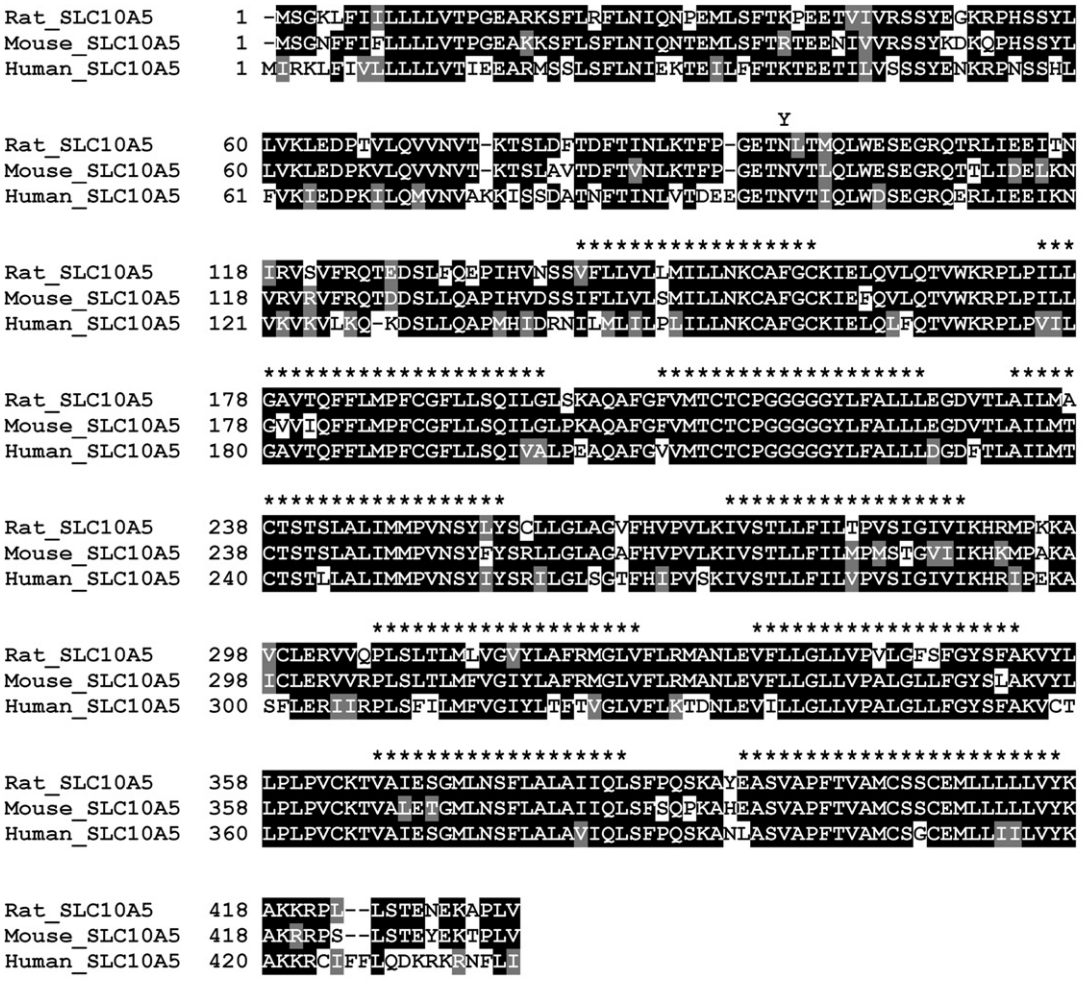

Fig. 1. Amino acid sequence alignment of the human, rat, and mouse SLC10A5 proteins. Multiple sequence alignment was conducted using the *ClustalW* algorithm and was visualized by BOXSHADE 3.21. Amino acid sequence identity is displayed with black shading; amino acid similarities are highlighted in grey. Gaps (-) are introduced to optimize alignment. Putative transmembrane regions of SLC10A5 were predicted by HMMTOP, TMpred, and TopPred calculations and are indicated by asterisks (*). One conserved putative *N*-glycosylation site exists in the N-terminus of the SLC10A5 proteins (marked by Y).

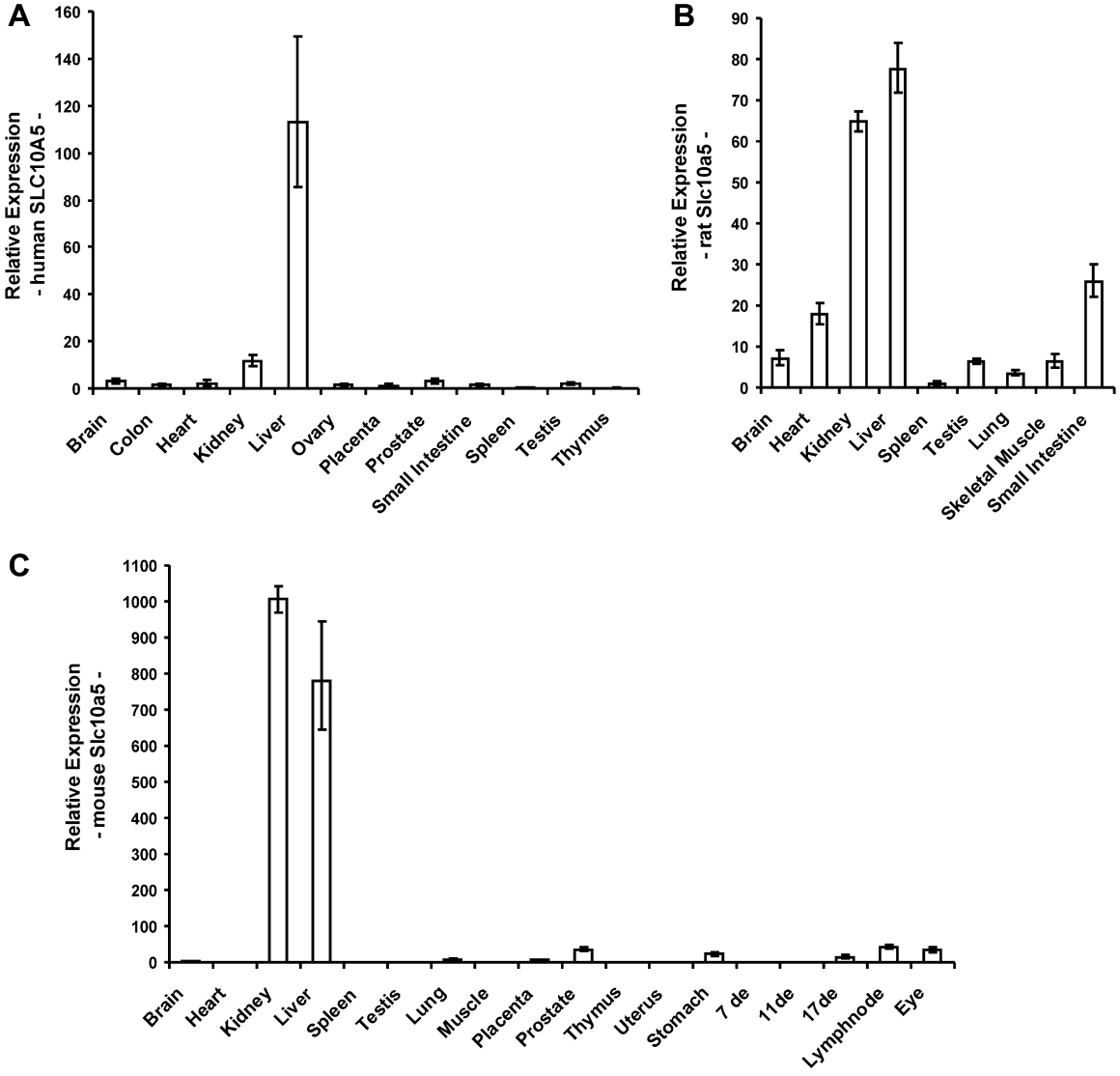

Fig. 2. Expression pattern of (A) human SLC10A5, (B) rat Slc10a5, and (C) mouse Slc10a5, analyzed by real-time quantitative PCR. Template cDNAs were derived from multiple tissue cDNA panels, which have been normalized to the mRNA expression levels of four different housekeeping genes. Relative expression was calculated by $2^{-\Delta\Delta C_T}$ transformation and represents x-fold higher SLC10A5/Slc10a5 expression in the respective tissue than in the spleen, which was the organ with the lowest SLC10A5/Slc10a5 expression. The data is presented as means ± SD of quadruplicate measurements. de, day embryo.

general, the detected mRNA levels were high in liver and kidney, but low in all other organs. This points to a tissue-specific expression pattern of SLC10A5/Slc10a5 that is identical across different species. The highest relative Slc10a5 expression in kidney and liver was detected in the mouse, where respectively 1000 and 800 times higher mRNA levels were detected than in the spleen, which was the tissue with the lowest Slc10a5 expression (Fig. 2C). Furthermore, it was found that SLC10A5 expression in humans is higher in liver than in kidney (Fig. 2A). Cellular localization of rat Slc10a5 expression was performed by *in situ* hybridization using specific digoxigenin-labeled Slc10a5 sense and antisense RNA probes. As shown in Fig. 3, rat Slc10a5 expression was detected in hepatocytes and renal proximal tubules.

For functional characterization of the SLC10A5 protein, we used SLC10A5-cRNA injected *X. laevis* oocytes and analyzed the transport of radiolabeled bile acids and sulfoconjugated steroid hormones in comparison to water-injected oocytes. In contrast to NTCP-expressing oocytes, which were used as the positive control, no transport activity was detected for taurocholate, cholate, estrone-3-sulfate, or dehydroepiandrosterone sulfate in the SLC10A5-expressing oocytes (Table 1). In order to analyze whether the lack of transport activity of SLC10A5 in the oocytes resulted from insufficient protein synthesis or membrane insertion, we attached the FLAG epitope to the C-terminal end of human, rat, and mouse SLC10A5 proteins and analyzed the appearance of the FLAG-tagged proteins in the oocytes by immunofluorescence detection. As shown in Fig. 4A, human, rat, and mouse SLC10A5-FLAG proteins were clearly detected in the oocyte's plasma membrane. The SLC10A5-FLAG proteins were also expressed in HEK293 cells, but

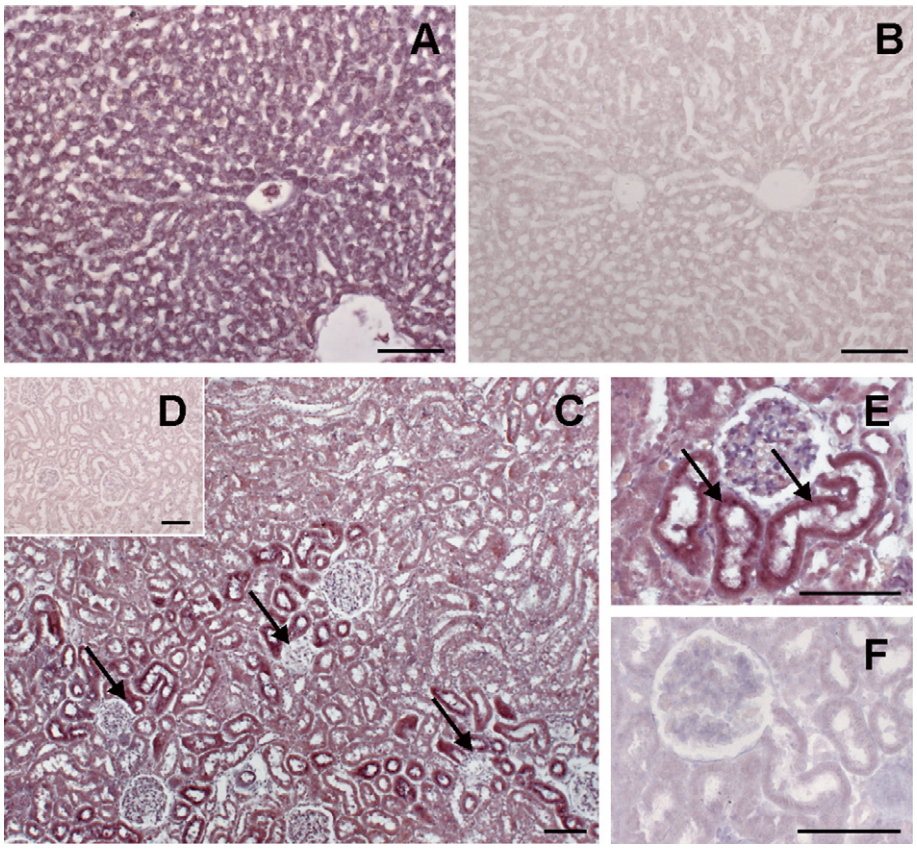

Fig. 3. *In situ* hybridization analysis of Slc10a5 mRNA expression in (A–B) rat liver and (C–F) rat kidney. Liver and kidney sections were hybridized with digoxigenin-labeled Slc10a5 antisense (A,C,E) and sense (B,D,F) probes. Slc10a5 expression was detected in hepatocytes and renal proximal tubules (indicated by arrows). Scale bar: 100 μm.

Table 1
Uptake studies with the human and mouse SLC10A5 proteins in *Xenopus laevis* oocytes

Test compound	Uptake (fmol/oocyte/60 min)	Ratio	Uptake (fmol/oocyte/60 min)	Ratio
	NTCP		Human SLC10A5	
Taurocholate (6.4 μM)	11470 ± 7526	468*	28.3 ± 6.8	1.2
Cholate (5 μM)	13132 ± 6612	81*	202 ± 57	1.2
Estrone-3-sulfate (20 nM)	134 ± 59	50*	3.8 ± 1.6	1.4
DHEAS (2 μM)	3061 ± 1454	13*	285 ± 86	1.2
	NTCP		Mouse SLC10A5	
Taurocholate (5 μM)	1383 ± 568	180*	8.7 ± 1.3	1.1
Estrone-3-sulfate (100 nM)	77.8 ± 12.8	6.0*	17.8 ± 4.8	1.4

Note: Values represent means ± SD of 10 separate oocyte measurements. Ratio represents uptake into carrier-cRNA injected oocytes divided by uptake into water-injected oocytes.
DHEAS: dehydroepiandrosterone sulfate.
* Significantly higher uptake into carrier-cRNA injected oocytes compared with water-injected oocytes ($p < 0.001$, one-way ANOVA with Bonferroni post hoc test).

showed also no transport activity for taurocholate and estrone-3-sulfate in this expression system (data not shown). HEK293 cells expressing the mouse SLC10A5-FLAG protein were used for radioimmunoprecipitation experiments with an anti-FLAG antibody. A specific band for the FLAG-tagged SLC10A5 protein was detected at 42 kDa which did not appear in the empty-vector transfected HEK293 cells. An additional faint band was visible at 84 kDa (Fig. 4B).

Discussion

The SLC10 Carrier Family is well established as the "sodium/bile acid cotransporter family" [13]. The founding members of this carrier family, NTCP (SLC10A1) and ASBT (SLC10A2), were cloned at the beginning of the 1990s by expression cloning in *X. laevis* oocytes [1,2] and showed high species-independent expression in liver and intestine, respectively. Besides SLC10A1 and SLC10A2,

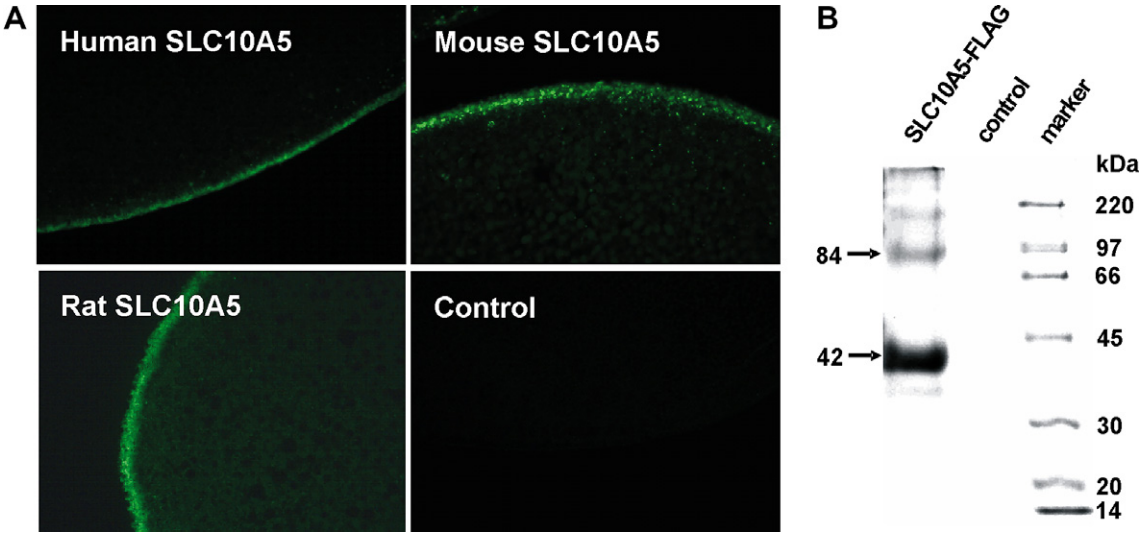

Fig. 4. Expression of SLC10A5-FLAG fusion proteins in (A) *Xenopus laevis* oocytes and (B) HEK293 cells. (A) The human, mouse and rat SLC10A5-FLAG tagged proteins were detected in the plasma membrane of SLC10A5-FLAG-cRNA injected *Xenopus laevis* oocytes by immunofluorescence detection using an anti-FLAG antibody and Alexa Fluor 488. Water-injected oocytes were used as the negative control. (B) HEK293 cells transfected with the mouse Slc10a5-FLAG-pcDNA5 vector or empty vector (control) were used for radioimmunoprecipitation with an anti-FLAG antibody. The samples were separated by gel electrophoresis on a 12% SDS–polyacrylamide gel and exposed to standard X-ray film for visualization.

the P3 gene also belongs to the SLC10 carrier family. This gene was identified in 1988, even before NTCP and ASBT were discovered and was retrospectively included into the SLC10 family because of certain sequence homologies [13,14]. The P3 gene, now referred to as SLC10A3, has a CpG island in its promoter region and shows a broad tissue expression pattern [15]. Although known for more than 15 years, still no specific function has been found for SLC10A3. More recently, four new members of the SLC10 family (SLC10A4–SLC10A7) were identified by homology-based sequence prediction from the sequenced human, rat, and mouse genomes [13,14]. One of these new members, the sodium-dependent organic anion transporter SOAT (SLC10A6), transports sulfoconjugated steroid hormones such as estrone-3-sulfate, dehydroepiandrosterone sulfate, and pregnenolone sulfate but not bile acids such as taurocholate or cholate [11,12]. In contrast, SLC10A4 and SLC10A7 remain orphan carriers [14].

In this paper, we report for the first time on the cloning and characterization of SLC10A5, which shows its highest sequence identity to SLC10A3. Both SLC10A3 and SLC10A5 derived from a common ancestor gene and both genes exhibit only one coding exon in humans, rat, and mouse [14]. The deduced amino acid sequences of the human, rat, and mouse SLC10A5 proteins show high sequence identities of >70%. Further SLC10A5-related sequences were identified by Ensembl orthologue predictions (Ensembl release 43) from cattle (Btau 3.1) and chimp (PanTro 2.1) and also exhibit high sequence identities to the human SLC10A5 protein (76% and 98%, respectively). As with NTCP and ASBT, SLC10A5/Slc10a5 showed a distinct tissue-specific expression pattern in humans, rat, and mouse, with its highest expression in liver and kidney. In rat, Slc10a5 expression was further localized by *in situ* hybridization to hepatocytes and renal proximal tubules. Whether SLC10A5 is involved in the hepatic and renal transport of bile acids or other solutes, could not be established in this study and needs further investigation.

The bile acid carriers NTCP and ASBT in their deglycosylated forms showed apparent molecular weights of 33.5 kDa [4] and 35 kDa [16], respectively, which is lower than the predicted molecular masses of the non-glycosylated proteins. Similar results were found for the FLAG-tagged mouse SLC10A5 protein that showed after radioimmunoprecipitation a specific band at 42 kDa which is 7 kDa smaller than its predicted mass of 49 kDa (48 kDa for the SLC10A5 protein + 1 kDa for the FLAG epitope). In addition to the 42 kDa band, we also found a faint band of higher molecular weight at 84 kDa that possibly represents a N^{96}-glycosylated form of the mouse SLC10A5 protein or a larger apparent protein aggregate, as was also reported for NTCP and ASBT [3,4,17].

Although expressed in the plasma membrane of *X. laevis* oocytes, SLC10A5 did not mediate uptake of established substrates of the other SLC10 carriers NTCP, ASBT, and SOAT, i.e., bile acids such as taurocholate and cholate and sulfoconjugated steroid hormones such as estrone-3-sulfate and dehydroepiandrosterone sulfate [6,7,12]. Although we have not yet proven that SLC10A5 is a bile acid carrier, we assume, based on the liver-specific and kidney-specific expression pattern and the close phylogenetic relationship to NTCP and ASBT, that SLC10A5 is still a carrier protein but for other kinds of bile acids or other organic solutes that were not tested in this study.

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