Cloning and Functional Characterization of Human Sodium-dependent Organic Anion Transporter (SLC10A6)*

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We have cloned human sodium-dependent organic anion transporter (SOAT) cDNA, which consists of 1502 bp and encodes a 377-amino acid protein. SOAT shows 42% sequence identity to the ideal apical sodium-dependent bile acid transporter ASBT and 33% sequence identity to the hepatic Na⁺/taurocholate-cotransporting polypeptide NTCP. Immuno-precipitation of a SOAT-FLAG-tagged protein revealed a glycosylated form at 46 kDa that decreased to 42 kDa after PNGase F treatment. SOAT exhibits a seven-transmembrane domain topology with an outside-to-inside orientation of the N-terminal and C-terminal ends. SOAT mRNA is most highly expressed in testis. Relatively high SOAT expression was also detected in placenta and pancreas. We established a stable SOAT-HEK293 cell line that showed sodium-dependent transport of dehydroepiandrosterone sulfate, estrone-3-sulfate, and pregnenolone sulfate with apparent $K_m$ values of 28.7, 12.0, and 11.3 $\mu$M, respectively. Although bile acids, such as taurocholic acid, cholic acid, and chenodeoxycholic acid, were not substrates of SOAT, the sulfoconjugated bile acid taurolithocholic acid-3-sulfate was transported by SOAT-HEK293 cells in a sodium-dependent manner and showed competitive inhibition of SOAT transport with an apparent $K_i$ value of 0.24 $\mu$M. Several nonsteroidal organosulfates also strongly inhibited SOAT, including 1-(ω-sulfooxyethyl)pyrene, bromosulfophthalein, 2- and 4-sulfooxymethylpyrene, and α-naphthylsulfate. Among these inhibitors, 2- and 4-sulfooxymethylpyrene were competitive inhibitors of SOAT, with apparent $K_i$ values of 4.3 and 5.5 $\mu$M, respectively, and they were also transported by SOAT-HEK293 cells.

SLC10 (solute carrier family 10) is well established as the “sodium bile acid cotransporter family” (1). The first member of this transporter family, the Na⁺/taurocholate-cotransporting polypeptide (Ntcp; Slc10a1), was identified in 1990 by expression cloning from rat liver (2) and is exclusively expressed at the sinusoidal membrane of hepatocytes (3, 4). Three years later, its intestinal counterpart was cloned from a hamster intestinal cDNA library and was named apical sodium-dependent bile acid transporter (Asbt; SLC10a2) (5). In contrast to the basolateral localization of Ntcp, Asbt is highly expressed at the apical brush border membrane of enterocytes of the terminal ileum (6). Although sequence identity between NTCP and ASBT is quite low (at 35%), both carriers transport conjugated bile acids with high affinity (7–11).

Due to their transport characteristics and expression pattern, NTCP and ASBT are important factors for the maintenance of the enterohepatic circulation of bile acids mediating the first step in the cellular uptake of bile acids through the membrane barriers in the liver (NTCP) and intestine (ASBT). Since the bile acid reflux from the intestine is a major negative regulator of the de novo bile acid synthesis from cholesterol in the liver, ASBT is a promising drug target for cholesterol-lowering therapy (12). In fact, several compounds were able to significantly lower plasma cholesterol levels and prevent atherosclerosis in animal studies, and currently they are being tested in clinical trials (13).

Recently, four new members of the SLC10 family were discovered and referred to as SLC10A3, SLC10A4, SLC10A5, and sodium-dependent organic anion transporter (SOAT; SLC10A6) (14). SLC10A3 (P3) was cloned from placenta and teratocarcinoma cDNA libraries in 1988, before NTCP and ASBT had been discovered, and showed broad tissue expression (15). The second orphan transporter SLC10A4 seems to be predominantly expressed in the central nervous system and shares a common ancestor gene with NTCP. In contrast, SLC10A5 shows high expression in the liver, kidney, and intestine, which is very similar to the expression pattern of ASBT (14). Until now, however, these orphan transporters have not been subjected to intensive experimental expression analysis, and there is no published data indicating that they have any function as solute carriers. Finally, in 2004, we cloned rat Soat, which showed the highest phylogenetic relationship to ASBT but did not transport taurocholate (16). In this paper, we report on the cloning, membrane topology, and expression of human SOAT and also provide its functional characterization in stably transfected human embryonic kidney (HEK293) cells. Besides sulfoconjugated steroid hormones, SOAT also transports tauroliothcholic acid-3-sulfate and sulfoconjugated pyrenes.
EXPERIMENTAL PROCEDURES

Materials and Chemicals—All of the chemicals, unless otherwise stated, were from Sigma. Glycolithocholic acid was from Calbiochem. Phenylnsulfate, hydroquinone sulfate, α-naphthylsulfate, 1-(ω-sulfooxyethyl)pyrene (1ω-SEP), 2-ω-sulfooxymethylpyrene (2-SMP), 4-sulfooxymethylpyrene (4-SMP), and 5-sulfooxymethylfuluraf were prepared from the corresponding hydroxyl compounds and sulfuric acid in dimethylformamide using dicyclohexylcarbodimide as the condensing agent, as described in detail elsewhere (17, 18).

Radiochemicals—[3H]Dehydroepiandrosterone sulfate ([3H]DHEAS, 60 Ci/mmol), [3H]estrone-3-sulfate ([3H]E1S, 57 Ci/mmol), [3H]digoxin (24 Ci/mmol), and [3H]taurocholic acid (3.5 Ci/mmol) were purchased from PerkinElmer Life Sciences. [14C]Cholic acid (55 mCi/mmol), [14C]cholic acid (3.5 Ci/mmol) were purchased from PerkinElmer Life Sciences. [3H]Dehydroepiandrosterone sulfate ([3H]DHEAS, 60 Ci/mmol), [3H]estrone-3-sulfate ([3H]E1S, 57 Ci/mmol), [3H]taurocholic acid (3.5 Ci/mmol), [3H]pregnenolone-3-sulfate ([3H]PREGS, 20 Ci/mmol), [3H]dihydroxytestosterone (76 Ci/mmol), [3H]estradiol-17β-d-glucuronide (44 Ci/mmol), [3H]dehydroepiandrosterone (54 Ci/mmol), and [3H]ouabain (23 Ci/mmol) were obtained from PerkinElmer Life Sciences. [3H]Taurolithocholic acid 3-sulfate ([3H]TLCs, 24.1 Ci/mmol) was generously donated by Werner Kramer (Sanofi-Aventis, Frankfurt am Main, Germany).

Cloning of Human SOAT cDNA—Using BLAST searches of the human genome with the cDNA sequences of the six coding exons of rat Soat (Slc10a6) (GenBankTM accession number AJ583502), we obtained matches with six genomic sequence fragments on human chromosome 4q21. These sequences were used for an RT-PCR-based strategy to obtain the full open reading frame cDNA sequence of human SOAT. The following oligonucleotide primers were designed, including SaclI/Xbal restriction sites for PCR amplification: forward primer, 5′-agc ggc atg tat gtg-3′; and reverse primer, 5′-atc cct tgt gtg cct gac cat tc-3′. Although it has a relatively low expression in this organ (see below), human SOAT was initially cloned from the adrenal gland. RT-PCR was performed from 1 μg of human adrenal gland poly(A)-SEP, 1-μg RNA (BD Clontech) using the Expand High Fidelity PCR System (Roche Applied Science) according to the following thermocycling conditions: one cycle of 94°C for 5 min; five cycles of 94°C for 30 s and 72°C for 1 min; five cycles of 94°C for 30 s and 70°C for 1 min; 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min; and final extension of 72°C for 10 min. After the amplification reaction, samples were held at 4°C until analysis. An aliquot of the PCR product was electrophoresed on an agarose gel. The amplicon of 1152 bp was excised form the gel and digested for 90 min at 37°C with SacII and Xbal. In order to obtain a SOAT-pBluescript plasmid, the sticky ended SOAT cDNA fragment was directionally ligated downstream from a T3 promoter into the pBluescript vector (Stratagene), which was indiscriminated with the respective restriction enzymes (SacII and XbaI). Three different clones were sequenced on both strands, and the cDNA sequence was deposited in the GenBankTM data base under GenBankTM accession number AJ583502. In order to confirm transcription of the full-length SOAT mRNA sequence also on organs with high SOAT expression (i.e. testis, placenta, and pancreas) (see below), RT-PCR was also performed on human testis, placenta, and pancreas cDNAs (BD Clontech) as described above, and PCR fragments were verified by DNA sequencing. To confirm transport activity of human SOAT, the SOAT-pBluescript plasmid was used for transport experiments with [3H]DHEAS and [3H]E1S in Xenopus laevis oocytes as described in detail previously (16).

Identification of SOAT cDNA Ends by Rapid Amplification of cDNA Ends (RACE)-PCR—In order to obtain the full-length SOAT mRNA transcript, we employed the GeneRacer method based on RNA ligase-mediated and oligonucleotide-capping RACE according to the manufacturer's protocol (Invitrogen). Reverse transcription of 1 μg of testis RNA (BD Clontech) was performed with the GeneRacer Oligo dT Primer 5′-gct gag ctg ctg gaa caa ttg gtt act ctc ctc tcc ctc tcc tct gaa ctg ttg-3′ in a volume of 20 μl using SuperScript III Reverse Transcriptase (Invitrogen). Initial 3′- and 5′-RACE reactions were performed using the gene-specific primers 5′-ggc agc tcc tcc tct caa ggt tgg tgg tgg ccc act g-3′ for 5′-RACE amplification and 5′-aat tac tgt tgt ggt gcc cat tc-3′ for 3′-RACE amplification. For each 50-μl reaction, 1 μl of cDNA, 0.5 μl of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 6 μl of MgCl2 (25 mm) were used, and amplification was performed under the following thermocycling conditions: 94°C for 5 min; 5 cycles of 94°C for 30 s and 72°C for 1 min; 5 cycles of 94°C for 30 s and 70°C for 1 min; 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min; and final extension of 72°C for 10 min. To increase the yield and specificity of the RACE products, additional nested PCR was performed using the nested primers 5′-gct gag ctg ctg gaa caa tgg ggt tgt tgg cct gcc ttg ggt gac cat tc-3′ for the nested 3′-RACE reaction and 5′-cct gtt ggc ttt ggt ggt tct ggt-3′ for the nested 5′-RACE reaction. The nested 3′- and 5′-RACE reactions were performed using the GeneRacer protocol (Invitrogen). Three individual clones were sequenced on both strands for each PCR fragment.

Establishment of the SOAT-HEK293 Cell Line—The recombinant human cell line T-REx SOAT-HEK293 was made using the Flp-In expression system and the commercially available Flp-In T-REx 293 host cell line according to the manufacturer's instructions (Invitrogen). Flp-In T-REx 293 cells contain a single, stably integrated Flp recombinase target (FRT) site at a transcriptionally active genomic locus, which is maintained by selection for zeocin resistance and ensures high-level gene expression from a target-integrated Flp-In expression vector. Brieﬂy, SOAT cDNA spanning the whole open reading frame was subcloned from SOAT-pBluescript vector into the Flp-In

2 The abbreviations used are: 1ω-SEP, 1-ω-sulfooxymethylpyrene; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; E1S, estrone-3-sulfate; DHEAS, dehydroepiandrosterone sulfate; PREGS, pregnenolone sulfate; 2-SMP, 2-sulfooxymethylpyrene; 4-SMP, 4-sulfooxymethylpyrene; 1TMD, transmembrane domain; RACE, rapid amplification of cDNA ends.
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pcDNA5/FRT/TO expression vector carrying the FRT site and the hygromycin resistance gene (Invitrogen). In the generated vector, further referred to as SOAT-pcDNA5, SOAT cDNA is under the control of the cytomegalovirus promoter and the tetracycline operator sequences (tetO2). In addition to the FRT site, Flp-In T-Rex 293 cells stably express the tetracycline repressor, which is maintained by selection for blasticidin resistance. In the absence of tetracycline, tetracycline repressor effectively binds to the tetO2 sequence and blocks SOAT transcriptions from the cytomegalovirus promoter. In order to establish the SOAT-HEK293 cell line, the SOAT-pcDNA5 construct was cotransfected with the Flp recombinase expression vector pOG44 into Flp-In T-Rex 293 host cells by Fugene 6 transfection reagent according to the manufacturer’s protocol (Roche Applied Science). Upon cotransfection, the SOAT coding sequence was integrated into the genome of the Flp-In HEK293 cells via Flp recombinase-mediated homologous recombination at the FRT site. Stable clones containing the SOAT open reading frame sequence under control of the cytomegalovirus/tetO2 hybrid promoter were selected by culturing in selective medium containing 150 μg/ml hygromycin and 50 μg/ml blasticidin. After 10–14 days, single clones were isolated from the remaining cell pool using cloning cylinders and tested for sodium-dependent [3H]DHEAS transport. The best transporting cell clone (further referred to as SOAT-HEK293) was selected and used for further experiments. SOAT-HEK293 cells were maintained in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal calf serum (Sigma), L-glutamine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) (further referred to as standard medium) at 37 °C, 5% CO2, and 95% humidity.

Transport Studies in SOAT-HEK293 Cells—For transport studies, 12-well plates were coated with poly-d-lysine for better attachment of the cells. 1.25 × 105 cells/well were plated and grown under standard medium for 72 h. SOAT expression was induced by preincubation with tetracycline (1 μg/ml). SOAT nonexpressing control cells (Flp-In HEK293 cells) were not pretreated with tetracycline. Before starting the transport experiments, cells were washed three times with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 7.3 mM Na2HPO4, pH 7.4, 37 °C) and preincubated with sodium transport buffer containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.8 mM CaCl2, and 20 mM HEPES, adjusted to pH 7.4. When transport assays were performed in sodium-free transport buffer, sodium chloride was substituted with choline chloride, sodium gluconate, and potassium gluconate. Uptake experiments were initiated by replacing the preincubation buffer by 500 μl of transport buffer containing the radiolabeled test compound and were performed at 37 °C. For inhibition studies, SOAT-HEK293 cells were preincubated with transport buffer containing the inhibitor compound for 30 s. Then transport measurements were started by adding the radiolabeled substrate at 37 °C. Transport and inhibition assays were terminated by removing the transport buffer and washing five times with ice-cold PBS. Cell monolayers were lysed in 1 N NaOH with 0.1% SDS, and the cell-associated radioactivity was determined in a liquid scintillation counter. The protein content was determined according to Lowry using aliquots of the lysed cells with bovine serum albumin as a standard (19).

Uptake Studies with 2-SMP and 4-SMP—Cells were seeded in 24-well plates (2 × 105 cells in 1 ml of medium/well) 2 days before the experiment started. SOAT-HEK293 cells were incubated in Ringer’s solution (130 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 20 mM HEPES, 1 mM NaH2PO4, and 18 mM glucose, pH 7.4) or an equimolar solution in which sodium was replaced by choline in the presence of 10 μM 2-SMP or 4-SMP for 15 min at 37 °C. After aspiration of the transport solution and three washes with ice-cold Ringer’s solution, cells were lysed with 0.25 ml of 1 N NaOH. After neutralization with 0.25 ml of 1 N HCl and protein precipitation with 1 ml of acetic acid, aliquots (usually 10 μl) of the supernatant were injected into HPLC using a Shimadzu SIL-M10 A vp autosampler. Samples were separated using a Shimadzu SLC-10 A vp delivery system equipped with a Phenomenex Gemini C18 column (250 × 3 mm; 5 μm). The eluent was methanol containing 20% water and 0.05% triethylamine (v/v). The flow rate was 0.2 ml/min. 2-SMP and 4-SMP were quantified from the fluorescence signal (λem, 334 nm, λex, 392 nm) using a Shimadzu SPD-M10 A vp detector.

Expression of SOAT-FLAG-tagged and ASBT-FLAG-tagged Proteins—A FLAG-tagged SOAT protein was generated by insertion of the FLAG-peptide (DYKDDDDK) to the C-terminal end of SOAT by QuikChange site-directed mutagenesis (Stratagene) of the SOAT-pcDNA5 construct. The following oligonucleotide sense and antisense primers were used: 5′-cat ttc atg cta gga tga ata cca gga cgg gcc ctc tgg cag cta g-3′ sense and 5′-cca gtc atg ctc tgg cag ctc tgg cta ctc tgg ctc tgc atc ttc ctc ccc ctt cgg ctc atc tgc atg cca gga cgg cgc ctc tgg cag cta g-3′ antisense. Correct clones were selected by DNA sequencing. Furthermore, an ASBT-FLAG fusion protein was generated for comparative analysis. Briefly, the full open reading frame of human ASBT was amplified by RT-PCR from 1 μg of small intestine poly(A)+ RNA (BD Clontech). Gene-specific oligonucleotide primers were used containing SacII/XbaI restriction sites (5′-gcc gcc ggc ctc ctc cta g-3′ forward and 5′-gta g-3′ reverse), and PCR amplification was performed using the Expand High Fidelity PCR System (Roche Applied Science) according to the following touchdown schedule: 1 cycle of 94 °C for 2 min; 10 cycles of 94 °C for 15 s, 65 °C for 30 s minus 0.5 °C each cycle, and 72 °C for 1 min; 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min plus 10 s each cycle. The PCR product of the expected size was gel-purified and digested for 90 min at 37 °C with SacII and XbaI. The sticky ended cDNA fragment was directionally ligated by RT-PCR from 1 μg of small intestine poly(A)+ RNA (BD Clontech). Gene-specific oligonucleotide primers were used containing SacII/XbaI restriction sites (5′-gcc gcc ggc ctc ctc cta g-3′ forward and 5′-gta g-3′ reverse), and PCR amplification was performed using the Expand High Fidelity PCR System (Roche Applied Science) according to the following touchdown schedule: 1 cycle of 94 °C for 2 min; 10 cycles of 94 °C for 15 s, 65 °C for 30 s minus 0.5 °C each cycle, and 72 °C for 1 min; 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min plus 10 s each cycle. The PCR product of the expected size was gel-purified and digested for 90 min at 37 °C with SacII and XbaI. The sticky ended cDNA fragment was directionally ligated downstream from a T3 promoter into the pBluescript vector (Stratagene), which was digested with SacII and XbaI. Sequence verification was done according to the reference sequence with GenBank™ accession number NM_000452. Transport activity of ASBT was confirmed by transport experiments in X. laevis oocytes with [3H]taurocholic acid as the test compound (see above). For transfection of Flp-In
HEK293 cells, the ASBT open reading frame sequence was subcloned into the Flp-In pcDNA5/FRT/TO expression vector, and the FLAG epitope was inserted at the C-terminal end of ASBT by QuikChange site-directed mutagenesis as described above for SOAT. The following oligonucleotide primers were used: 5’-cag cct gcg gac aag gat tac aag gat gag gac gat aag tag cta tct cga gtc-3’ sense and 5’-gac tcc aga tgg cta ctt atc gtc atc atc ctt atc ctt ttc gtc agg tgg-3’ antisense. The SOAT-FLAG-pcDNA5 and ASBT-FLAG-pcDNA5 constructs were verified by DNA sequencing, and positive clones were used for further experiments.

**Immunoprecipitation and Deglycosylation of the FLAG-tagged Proteins**—Flp-In T-Rex 293 cells were seeded in 6-well plates coated with poly-D-lysine at a density of 1.0 × 10⁶ cells/well in antibiotic-free DMEM/F-12 medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. On the following day, the cells were transfected with 4 μg of SOAT-FLAG-pcDNA5 and ASBT-FLAG-pcDNA5 vector DNA or with 4 μg of pcDNA5 alone (control) by Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen). After 4 h, the medium was changed to standard medium, and expression of the FLAG-tagged proteins was induced by tetracycline treatment (1 μg/ml). On the next day, the cells were washed with PBS and starved in methionine-free and cysteine-free DMEM medium (Sigma) supplemented with 4 mM L-glutamine and 1 μg/ml tetracycline for 1 h. Subsequently, 70 μCi of L-[³⁵S] in vitro cell labeling mix (Amersham Biosciences) was added, and the cells were incubated for an additional 6 h at 37 °C, 5% CO₂, and 95% humidity. The cells were washed with ice-cold PBS and incubated in 500 μl of ice-cold radioimmunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 8.0), 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholic acid, 0.1% (w/v) SDS, and protease inhibitor mixture (Sigma) for 5 min under shaking. Cell lysates were transferred to a microcentrifuge tube and incubated for an additional 30 min under rotation at 4 °C. To remove any cell debris, the samples were centrifuged for 15 min at 4 °C, and the supernatant was transferred to a fresh tube. Immunoprecipitation was performed by incubation with 5 μg of the monoclonal mouse anti-FLAG antibody (Sigma) under rotation for 1 h at 4 °C. Subsequently, 100 μl of protein A-Sepharose (Sigma; 25% suspension in radioimmunoprecipitation buffer) was added, and samples were incubated under rotation. After 1 h, Sepharose beads were precipitated by centrifugation and washed three times with ice-cold radioimmunoprecipitation buffer. For deglycosylation with PNGase F, the protein A-Sepharose beads were resuspended in 1× glycoprotein denaturation buffer and boiled for 10 min, and the eluted proteins were incubated overnight at 37 °C with 1000 units of PNGase F in 1× G7 reaction buffer supplemented with 1% Nonidet P-40 (New England Biolabs). Nondeglycosylated samples were equally processed but not incubated with PNGase F. All samples were mixed with the same amount of 2× Laemmli buffer containing 10% β-mercaptoethanol and boiled for 10 min. Finally, deglycosylated and nondeglycosylated samples were separated by 12% SDS-PAGE. The gel was fixed in 30% methanol, 10% acetic acid (v/v) for 30 min and soaked in Amplify Fluorographic reagent (Amersham Biosciences) for 30 min. Dried gels were exposed to Eastman Kodak Co. BioMax MR film (Sigma) at −80 °C.

**Antibody Preparation**—The SOAT-(2–17) antibody was raised in rabbits against amino acid residues 2–17 of the deduced SOAT sequence (RANCSSSSACPNSSE). The synthetic peptide was coupled via the carboxyl-terminal glutamic acid residue to keyhole limpet hemocyanin and used to immunize two rabbits (Eurogentec). Antigenicity of the rabbit serum was confirmed by enzyme-linked immunosorbent assay analysis using the synthetic peptide as the antigen. A second SOAT-(349–364) antibody was raised against amino acid residues 349–364 at the C terminus of SOAT. However, this antibody showed no immunoreactivity against the synthetic peptide in enzyme-linked immunosorbent assay experiments and was not applicable (Eurogentec).

**Immunofluorescence Microscopy of SOAT-HEK293 Cells**—SOAT-HEK293 cells were grown on poly-D-lysine-coated glass coverslips to ~80% confluence in standard medium. SOAT expression was induced by tetracycline treatment (1 μg/ml) for at least 24 h. Noninduced control cells were equally processed but were not incubated with tetracycline. On the next day, cells were washed three times with PBS and then incubated with the SOAT-(2–17) rabbit antibody (1:10) for 1 h at room temperature without paraformaldehyde treatment. After rinsing and three times washing with PBS, the cells were incubated for 1 h at room temperature with the mouse fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody at 1:200 dilution (Sigma). After a final washing procedure, the cells were covered with a DAPI/methanol solution containing 1 μg/ml DAPI and incubated for 5 min at 8 °C. The cells were washed with methanol/acetic (1:1), air-dried, and mounted on slides with Mowiol mounting medium. Fluorescence imaging was performed on a Leica DM6000B fluorescence microscope. Captured files were analyzed with the Leica FW4000 fluorescence work station software.

**Immunofluorescence Microscopy of SOAT-FLAG-transfected HEK293 Cells**—For transient transfection, Flp-In HEK293 cells were seeded in 24-well plates at a density of 2.5 × 10⁵ cells/well on poly-D-lysine-coated glass coverslips and grown to ~80% confluence in antibiotic-free DMEM/F-12 medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. Cells were transfected with 1 μg of SOAT-FLAG-pcDNA5 vector DNA by Lipofectamine 2000 reagent (Invitrogen). The parental pcDNA5 vector lacking any insert was used as control. After 4 h, the medium was changed to standard medium, and the transfected cells were induced by tetracycline (1 μg/ml) for at least 24 h. After fixation with 2% paraformaldehyde in PBS for 15 min at 4 °C, the cells were washed twice with PBS and incubated with 20 mM glycerine in PBS for 5 min. Subsequently, the cells were permeabilized for 5 min in PBT buffer (PBS containing 0.2% Triton X-100 and 20 mM glycerine). Nonpermeabilized cells were not treated with PBT and were used for outside epitope localization. The cells were placed in blocking solution PBSG (1% bovine serum albumin and 4% normal goat serum in PBS) for 30 min at room temperature and incubated with the rabbit anti-FLAG antibody (Sigma) at a 1:40,000 dilution in PBSG overnight at 4 °C. The cells were washed three times with
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PBS and incubated with the goat Cy3-conjugated anti-rabbit IgG antibody (Jackson Immunoresearch) at 1:800 in PBS for 1 h at room temperature. After triple washing with PBS, nuclei were stained by incubation with a DAPI/methanol solution containing 0.2 μg/ml DAPI for 5 min at room temperature. The cells were washed with methanol, air-dried, and mounted on slides with Mowiol mounting medium. Fluorescence imaging was performed as described above.

Real Time Quantitative PCR—Relative SOAT expression analysis was performed with ABI PRISM 7300 technology using human multiple tissue cDNA panels (BD Clontech) and cDNA synthesized from human adrenal gland and human mammary gland RNAs (BD Clontech). PCR amplification was achieved with TaqMan Gene Expression Assays Hs01399354_m1 for human SOAT (SLC10A6) covering exon boundary 5–6 and Hs99999903_m1 for human β-actin (Applied Biosystems). Expression data of β-actin in each tissue were used as endogenous control. For each tissue, quadruplicate determinations were performed in a 96-well optical plate for both targets (SOAT and β-actin) using 2.5 μl of cDNA, 1.25 μl of TaqMan Gene Expression Assay, 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), and 8.75 μl of water in each 25-μl reaction. The plates were heated for 10 min at 95 °C, and subsequently 45 cycles of 15 s at 95 °C and 60 s at 60 °C were applied. Relative SOAT expression (ΔΔCT) was calculated by subtracting the signal threshold cycle (CT) of β-actin from the CT value of SOAT. Subsequently, for each tissue, ΔΔCT values were calculated by subtracting brain ΔCT (set as calibrator) from the ΔCT of each individual tissue and transformed by the 2−ΔΔCT equation to show x-fold higher SOAT expression in the respective tissue.

Bioinformatics—The BLAST program available on the World Wide Web was used to identify SOAT-encoding sequences in the human genome. Multiple sequence alignments were conducted using the EB1 ClustalW algorithm, available on the World Wide Web, and alignment was visualized by BOXSHADE, version 3.21. Amino acid identity values were determined after pairwise optimal GLOBAL alignment with the BioEdit program, version 7.0.5.2 (20). For similarity calculations, the DAYHOFF similarity matrix was used. Membrane topology and putative membrane-spanning domains were determined by the following programs: TMHMM (21), PRED-TMR2 (22), MEMSAT (23), TMAP (24), TopPred II (25), TMpred (26), and HMMTOP (27). The NetN Glyc 1.0 program was used to predict N-linked glycosylation sites, and NetPhos 2.0 was used to predict potential phosphorylation sites in the SOAT protein (28).

Statistical Analysis—Statistical significance for uptake measurements with radiolabeled substrates was calculated using Student’s t test. Statistical analysis of more than two groups was performed by one-way analysis of variance, followed by post hoc testing (Dunnett). Kinetic data from experiments measuring the uptake of radiolabeled substrates were fit to the Michaelis-Menten equation by nonlinear regression analysis. Dixon plot analysis was used for Kᵢ calculations.

| TABLE 1 | Exon-intron organization of the human SLC10A6 gene on chromosomal locus 4q21.3 |
|----------|---------------------------------------------------------------------------|
| Exon     | Exon size (bp) | 5’-Splice donor | 3’-Splice acceptor | Intron size (kb) |
| 1        | 525            | TCTCAG/gtaag    | tttcag/CTTCAG      | 15.3            |
| 2        | 119            | ACATAG/tgctg    | ttctag/GTACAT     | 1.4             |
| 3        | 89             | CTCAAG/gtgag    | tttcag/ATTCGG      | 1.4             |
| 4        | 176            | GCAGAG/gtagag   | ttagag/GTCAG      | 2.4             |
| 5        | 158            | TGCAG/gtggt     | acctag/CATAC      | 1.5             |
| 6        | 435            | TCAATG          |                   |                 |

RESULTS

Cloning of Human SOAT—Based on the cDNA sequence of rat Soat, we used an RT-PCR-based approach to clone the full 1134-bp open reading frame of human SOAT (GenBank accession number AJ583520). The full-length SOAT transcript was obtained by RACE-PCR and revealed a 1502-bp cDNA, including 5′- and 3′-untranslated regions of 148 and 220 bp, respectively (GenBank accession number EF437223). The human SLC10A6 gene is located on chromosome 4 and is coded by six exons mapped in region 4q21.3. As summarized in Table 1, the lengths of the SOAT exons account for 525, 119, 176, 158, and 435 bp. All intron/exon boundaries were compatible with the canonical donor and acceptor consensus motifs. Each intron started with GT at the 5′-splice donor site and ended with AG at the 3′-splice acceptor site. The cloned SOAT cDNA sequence exactly matched the genomic sequence of Homo sapiens chromosome 4, reference assembly (GenBank accession number NC_000004). The length of the human SLC10A6 gene accounts for 25.8 kb.

The SOAT protein consists of 377 amino acid residues with a calculated molecular mass of 41.2 kDa. Fig. 1 shows the deduced amino acid sequence of human SOAT in alignment with human NTCP and ASBT. SOAT has a higher amino acid sequence identity/similarity to ASBT (42%/70%), compared with human NTCP (33%/63%). At the C-terminal and N-terminal sequence, potential outer N-glycosylation sites (residues Asn4, Asn14, and Asn157) and potential inner facing serine and threonine phosphorylation sites (residues Ser60, Ser126, Ser259, Thr310, Ser335, Thr336, Ser347, Ser358, Thr354, and Thr374) are present in the SOAT protein sequence. Further bioinformatic analyses revealed a positively charged cluster in the SOAT C terminus at positions 312–339 (net charge = +9) and a tandem repeat “LTIP” at residues 158–161 and 172–175.

SOAT Tissue Expression—The expression of SOAT in different human tissues was investigated by real time quantitative PCR (Fig. 2A). Very low SOAT expression was found in brain, colon, kidney, liver, ovary, prostate, small intestine, spleen, and thymus. Expression levels were low also in the adrenal gland, from which the SOAT was initially cloned. In contrast, SOAT was highly expressed in human testis, where
the SOAT mRNA levels detected were 678 times higher than in brain (the tissue with lowest SOAT expression). Relatively high SOAT expression was also observed in human placenta and pancreas, and moderate expression was detected in heart, lung, and mammary gland. Because it has been reported that an exon-2-skipped, alternatively spliced form of ASBT is expressed in certain rat tissues (29), we performed additional RT-PCR experiments covering the whole open reading frame of SOAT from human testis, placenta, and pancreas RNAs. As shown in Fig. 2B, unique PCR ampli-
cons were detected that migrated at the expected size of 1152 bp on the agarose gel without occurrence of shorter SOAT transcripts.

Transport Properties of Human SOAT—Functional character-
ization of human SOAT was performed in stably trans-
fected SOAT-HEK293 cells using the Flp-In T-REx expres-
sion system, where SOAT expression is under control of the
tetracycline-regulated cytomegalovirus/tetO2 hybrid pro-
moter. SOAT expression was induced in SOAT-HEK293
cells by pretreatment with tetracycline. Transport experi-
ments were performed with several radiolabeled steroid
compounds. SOAT-specific transport activity was not
detected with nonconjugated steroids (estrone and dehydro-
epiandrosterone), glucuronidated steroids (estradiol-17β-
-D-glucuronide and estrone-3β-D-glucuronide), bile acids
(taurocholic acid, cholic acid, chenodeoxycholic acid,
deoxycholic acid, and lithocholic acid), or heart glycosides
(ouabain and digoxin) (Table 2). However, SOAT-specific
transport in SOAT-HEK293 cells was observed for DHEAS,
estrone-3-sulfate (E1S), and pregnenolone sulfate (PREGS).
Further characterization of this transport addressed time
dependence, sodium dependence, and concentration
dependence. Fig. 3 shows the time profile for [3H]DHEAS uptake by
SOAT-expressing HEK293 cells and noninduced control cells. In
the absence of tetracycline, no signif-
ificant transport of [3H]DHEAS was observed. In contrast, SOAT-
expressing HEK293 cells showed 3-fold (30 s) to 9-fold (10 min)
higher [3H]DHEAS transport over the control cells. Using identical
amounts of [3H]DHEAS (200 nm), transport studies were also per-
formed in the absence of Na+ by equimolar substitution with chlo-
ine. In these experiments, sodium replacement completely abolished
SOAT-mediated DHEAS trans-
port. The initial uptake velocity of
[3H]DHEAS was also analyzed in SOAT-expressing HEK293 cells
and revealed linear uptake over
75 s at concentrations ranging from
0.5 to 100 μM DHEAS (Fig. 4).
Finally, the concentration de-
pendence of [3H]DHEAS, [3H]E1S,
and [3H]PREGS transport was examined. As shown in Fig. 5,
SOAT-specific uptake of these compounds showed saturation
kinetics and followed the Michaelis-Menten equation. Kinetic
parameters were determined by nonlinear regression analysis
and yielded Km values of 28.7 ± 3.9, 12.0 ± 2.3, and 11.3 ± 3.0
μM and Vmax values of 1899 ± 81, 585 ± 34, and 2168 ± 134
pmol/mg of protein/min for DHEAS, E1S, and PREGS,
respectively.

Ion Dependence of SOAT-mediated Transport—To further
investigate the sodium dependence of SOAT transport, we per-
fomed transport experiments with equimolar substitutions of
sodium chloride in the transport buffer by other cations and/or
anions. As shown in Table 3, lithium chloride and potassium
chloride maintained 36 and 23% of the transport function in
relation to sodium chloride, respectively. In contrast, choline
and N-methyl-D-glucamine could not maintain [3H]DHEAS
transport by SOAT. Replacement of chloride by gluconate in
the sodium-containing and potassium-containing transport
buffers revealed similar transport activities. This indicates that
SOAT transport is independent of chloride but highly depends
on the presence of sodium in the transport buffer.

Inhibition Studies with SOAT-HEK293 Cells—Inhibition
experiments were performed in SOAT-HEK293 cells to inves-
tigate the substrate selectivity of SOAT (Fig. 6). Cis-inhibitory
effects of the indicated compounds on SOAT-mediated uptake
of 2.5 μM [3H]DHEAS were examined at 25 μM concentrations.
Among the group of bile acids, the trihydroxylated bile acids
taurocholic acid, glycocholic acid, and cholic acid were no
inhibitors for SOAT-mediated [3H]DHEAS transport. In con-
trast, dihydroxylated bile acids inhibited SOAT transport
markedly, whereby inhibitory potency was higher for the
3αβ-7α; 3α-dihydroxylated bile acids than for the 3α,12α-dihy-
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droxyxoycholic acid, this bile acid is not a substrate of SOAT (Table 2). SOAT inhibition was also observed by the 3α-monohydroxylated bile acids lithocholic acid, glycolithocholic acid, and tauroliothocholic acids. Again, [3H]lithocholic acid was not transported by SOAT-HEK293 cells in direct transport experiments (Table 2). Finally, sulfoconjugated 3α-monohydroxylated bile acids were tested. These sulfated bile acids are structurally similar to the sulfated steroid hormones bearing an anionic sulfate moiety and a lipophilic steroid nucleus. At a 10-fold molar excess of unlabeled compounds, uptake of 2.5 μM [3H]DHEAS was reduced to less than 10% by tauroliothocholic acid-3-sulfate (TLCS), glycolithocholic acid-3-sulfate, and lithocholic acid-3-sulfate. TLCS, which was the most potent SOAT inhibitor among the group of sulfoconjugated bile acids, was also used for competitive inhibition experiments. Here, uptakes of 0.5 and 2.5 μM [3H]DHEAS by SOAT were inhibited by increasing concentrations of TLCS (Fig. 7C). An apparent $K_i$ value of 0.24 μM was determined from Dixon plot transformation; this is 2 orders of magnitude lower than the apparent $K_m$ value for DHEAS (i.e. 28.7 μM).

Furthermore, cis-inhibitory effects of the SOAT transport were examined with a set of xenobiotic organosulfates. At 25 μM concentrations, SOAT-mediated DHEAS transport was reduced to 4% by 1ω-SEP, to 18% by bromosulfophthalein, to 25% by 2-SMP and 4-SMP, and to 43% by α-naphthylsulfate. In the case of 2-SMP and 4-SMP, additional inhibition experiments were performed using 100 and 500 nM concentrations of the SOAT substrate E$_1$S (Fig. 7, A and B). $K_i$ values, determined from Dixon plots, were 4.3 and 5.5 μM for 2-SMP and 4-SMP, respectively. In contrast, other sulfoconjugated organic molecules had little or no inhibitory activity for SOAT transport at 25 μM concentrations. These included ethylsulfate, phenylsulfate, phenylethylsulfate, 2-propylsulfate, 5-sulfooxymethylfurfural, hydroquinone sulfate, 4-methylumbelliferon sulfate, and indoxylsulfate (Fig. 6). Furthermore, a series of differently substituted naphthyl derivatives were tested to discriminate whether the sulfate moiety can be replaced by other groups for SOAT inhibition. However, in contrast to α-naphthylsulfate, α-naphthylisothiocyanate, α-naphthylphosphate, and α-naph-

FIGURE 2. Expression of SOAT in various human tissues. SOAT tissue expression was analyzed by quantitative real time PCR analysis (A) and conventional PCR (B) using human multiple cDNA panels and cDNAs synthesized from human adrenal gland and mammary gland RNAs. Gene-specific primers and probes were used as outlined under “Experimental Procedures.” A, relative SOAT expression was calculated by the $2^{-\Delta C_t}$ method and represents SOAT expression that is x times higher in the respective tissue than in brain (set as calibrator). The values represent means ± S.E. of quadruplicate measurements. B, RT-PCR experiments covering the whole open reading frame of SOAT.

TABLE 2

| Uptake of various 3H-labeled and 14C-labeled compounds by SOAT-HEK293 cells | SOAT-HEK293 | Control | Ratio (SOAT/control) |
|-----------------------------|-------------|---------|---------------------|
| DHEAS (0.2 μM) | 13.1 ± 0.7 | 1.1 ± 0.04 | 12.2* |
| E$_1$S (0.2 μM) | 10.2 ± 0.5 | 0.8 ± 0.1 | 12.1* |
| PREGS (0.2 μM) | 146.1 ± 6.5 | 195.3 ± 3.7 | 7.5 |
| Estrone (1 μM) | 21.5 ± 0.5 | 21.7 ± 0.7 | 1.0 |
| Dehydroepiandrostosterone (1 μM) | 31.0 ± 1.3 | 33.4 ± 0.7 | 1.0 |
| Estradiol-17β-δ-glucuronide (1 μM) | 4.8 ± 0.6 | 4.4 ± 0.5 | 1.1 |
| Estrone-3β-δ-glucuronide (1 μM) | 17.3 ± 2.7 | 15.9 ± 2.5 | 1.1 |
| Taurocholic acid (1 μM) | 1.7 ± 0.3 | 1.8 ± 0.2 | 1.0 |
| Cholic acid (2.5 μM) | 20.5 ± 4.4 | 21.0 ± 2.4 | 1.0 |
| Chenodeoxycholic acid (2.5 μM) | 387.1 ± 37.9 | 414.6 ± 49.7 | 0.9 |
| Deoxycholic acid (2.5 μM) | 124.3 ± 4.9 | 124.3 ± 4.4 | 1.0 |
| Lithocholic acid (2.5 μM) | 281.4 ± 20.8 | 310.0 ± 11.0 | 0.9 |
| Ouabain (1 μM) | 1.5 ± 0.06 | 1.5 ± 0.1 | 1.0 |
| Digoxin (1 μM) | 6.2 ± 0.3 | 6.2 ± 0.1 | 1.0 |

* Uptake values by SOAT-HEK293 cells were significantly different from control cells with $p < 0.001$ (Student’s t test).
thylamine had no inhibitory effect on the DHEAS transport in SOAT-HEK293 cells (Fig. 6).

Transport of [3H]TLCS, 2-SMP, and 4-SMP in SOAT-HEK293 Cells—Since TLCS, 2-SMP, and 4-SMP competitively inhibited SOAT transport, we further investigated whether they are also substrates of SOAT. No radiolabeled compounds were available for 2-SMP and 4-SMP, so we determined the intracellular accumulation of 2-SMP and 4-SMP in the SOAT-HEK293 cells with an HPLC-based method using fluorescence detection. As shown in Fig. 7, D–G, 2-SMP, 4-SMP, and TLCS were transported in SOAT-expressing HEK293 cells with a ratio of about 2 over control cells. This transport was only observed if the experiments were performed in Na\textsuperscript{+}-containing transport buffer and was completely abolished if sodium was substituted by equimolar concentrations of choline, thus indicating that 2-SMP, 4-SMP, and TLCS were transported by SOAT in a sodium-dependent manner (Fig. 7, D and E). Fur-
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TABLE 3
Influence of equimolar substitutions of NaCl on [3H]DHEAS uptake by SOAT-HEK293 cells

Uptake of 200 nm [3H]DHEAS was measured at 37 °C in SOAT-HEK293 cells after preincubation with tetracycline (1 μg/ml). 142 mM NaCl was used in the transport buffer for the 100% control experiment and was substituted with equimolar concentrations of sodium gluconate, potassium gluconate, lithium chloride, potassium chloride, N-methyl-D-glucamine, and choline chloride. After 5 min, the transport medium was removed, and the cell monolayer was washed and subjected to radioactivity and protein measurements. The values represent means ± S.D. of two independent experiments, each with triplicate determinations.

| DHEAS uptake     | Percentage of control |
|------------------|-----------------------|
|                  | pmol/mg protein/5 min%|
| Sodium chloride (control) | 10.4 ± 0.5 | 100   |
| Lithium chloride | 3.8 ± 0.3 | 36%   |
| Potassium chloride | 2.4 ± 0.3 | 23%   |
| Choline chloride  | 0.4 ± 0.04 | 3.8%   |
| N-Methyl-D-glucamine | 0.3 ± 0.07 | 2.7%   |
| Sodium gluconate | 9.9 ± 0.8 | 95%   |
| Potassium gluconate | 1.6 ± 0.1 | 15%   |

* Uptake values after equimolar substitution of NaCl were significantly different from control experiments with p < 0.01 (one-way analysis of variance with Dunnett post hoc analysis).

thermore, 4-SMP transport was blocked by a 5-fold molar excess of E1S in the transport medium (Fig. 7F). In contrast, transport of 50 nm TLCS was inhibited by not less than 50 μM DHEAS (Fig. 7G), which is consistent with the lower Ki value of TLCS compared with 4-SMP.

Membrane Topology of Human SOAT—Analysis of the membrane topology of human SOAT was performed with different topology prediction programs. TMHMM, PREDTMR2, MEMSAT, TMAP, TopPred II (GES-scale), and TMPred proposed a membrane topology of SOAT with eight transmembrane domains and an extracellular location of the N-terminal and C-terminal domains (Fig. 8A). In contrast, HMMTOP analysis preferred a model with nine transmembrane domains, and TopPred II (KD-scale) calculated a seven-TMD topology. In all predictions, the N terminus of SOAT has an extracellular orientation and contains ~30 amino acid residues. This orientation is predicted due to a cluster of positively charged amino acid residues just downstream from TMD 1 (net charge of the N terminus = -4), net charge of the first intracellular loop = +3). The C terminus is inside in the seven-TMD and nine-TMD models but has an extracellular orientation in the eight-TMD topology (Fig. 8A). Similar discrepancies from in silico topology predictions were obtained for NTCP and ASBT. For these SLC10 carriers, experimental data clearly favored a seven-TMD topology. To determine whether a seven-TMD topology can be applied also for SOAT, we directly compared the hydrophobicity profiles of SOAT, ASBT, and NTCP in an overlay of the individual hydrophobicity plots. As shown in Fig. 8B, hydrophobicity values of SOAT and ASBT are nearly identical, indicating that both carriers show similar membrane topology. However, both proteins differ from the NTCP hydrophobicity pattern, particularly concerning amino acid residues 70–170, which represent transmembrane helices 2–4.

Localization of the N-terminal and C-terminal Domains of SOAT—Membrane expression and the C/N terminus orientation of human SOAT were analyzed in vitro in SOAT-HEK293 cells and HEK293 cells expressing the SOAT-FLAG fusion protein in which the FLAG motif (DYKD- DDDK) was attached to the C-ter-
minal end of SOAT. To confirm the extracellular orientation of the N terminus, we generated a SOAT antibody (SOAT-(2–17)) directed against the N-terminal 2–17 amino acids. Using this antibody, SOAT expression was analyzed in SOAT-HEK293 cells that were either induced or noninduced by tetracycline treatment and were kept under native (nonpermeabilized) conditions (Fig. 8 C). Fluorescence signals were only observed in the SOAT-expressing HEK293 cells, and no cell-associated fluorescence was detected in the noninduced control cells. Since SOAT-HEK293 cells were not fixed and not permeabilized for these experiments before incubation with the SOAT-(2–17) antibody, the N terminus of SOAT must be located in the extracellular compartment. In order to discriminate also the inside/outside orientation of the C terminus, we generated a second SOAT antibody, which was directed against amino acids 349–364 of the C terminus (SOAT-(349–364)). This antibody failed to show in vitro immunoreactivity against the SOAT-(349–364) peptide, so we decided to attach the FLAG epitope tag to the SOAT C terminus, which was then detected by using a commercial anti-FLAG antibody. A SOAT-FLAG-pcDNA5 construct was generated by site-directed mutagenesis and transfected into HEK293 cells to evaluate the accessibility of the C-terminal FLAG epitope by immunofluorescence microscopy under permeabilized and nonpermeabilized conditions. FLAG-directed fluorescence staining was only observed if the cells were permeabilized by Triton X-100, and it was undetectable in the nonpermeabilized cells (Fig. 8D). This clearly indicates a cytosolic orientation of the SOAT C terminus and excludes an eight-TMD topology.

Immunoprecipitation and Deglycosylation of the SOAT-FLAG Protein—SOAT-FLAG-pcDNA5-transfected HEK293 cells were also used for radiolabeling and immunoprecipitation experiments of the SOAT-FLAG protein with a monoclonal anti-FLAG antibody (Fig. 9). After separation of the precipitated cell extract, specific bands were detected at 46 and 42 kDa. The SOAT-FLAG protein consists of 377 + 8 amino acids with a predicted molecular mass of 41 kDa (SOAT) + 1 kDa (FLAG epitope). The higher apparent molecular mass of 46 kDa after immunoprecipitation was due to posttranslational modifications in the HEK293 cells. Since SOAT encodes three potential N-linked glycosylation sites, N-glycosylation of the SOAT-FLAG protein was examined by PNGase F digestion of the immunoprecipitated cell extract. As shown in Fig. 9, the apparent molecular mass of the SOAT-FLAG protein was decreased from 46 to 42 kDa after PNGase F incubation. This change in apparent molecular mass is consistent with the addition of at least one.
N-linked carbohydrate chain to any of the potential N-linked glycosylation sites of SOAT (Asn\(^4\), Asn\(^{14}\), and Asn\(^{157}\)). Based on these data, molecular masses of 45 and 41 kDa can be estimated for the untagged glycosylated and nonglycosylated SOAT proteins, respectively. For comparison, also the ASBT-FLAG protein was examined and also revealed a decrease of its apparent molecular mass from 40 to 37 kDa after PNGase F treatment, as has been previously reported (6, 7, 30).

**DISCUSSION**

**Transport Function of NTCP, ASBT, and SOAT**—In the past decade, the transport function of NTCP and ASBT were extensively studied in several cell systems and revealed transport of all physiological dihydroxylated and trihydroxylated bile acids with a preference for the taurine and glycine amidated conjugates versus the unconjugated forms (9–11, 31). SOAT is the third member of the SLC10 transporter family that we have functionally characterized in this paper. Transport studies in SOAT-HEK293 cells revealed no transport activity for taurocholic acid and cholic acid, thus indicating that SOAT, although belonging to the bile acid transporter family SLC10, is not a typical bile acid transporter, such as NTCP and ASBT. The latter also do not share identical substrate patterns. In contrast to ASBT, to which SOAT has closest homology, the substrate specificity of NTCP is not strictly limited to bile acids and also includes sulfocjugated steroid hormones, such as E\(_1\)S (9, 10). Additionally, it has been reported by Craddock and co-
orders of magnitude lower than the inhibited SOAT transport. These included 1-sulfocholate, 5-ethylsulfate, 2-propylsulfate, phenylsulfate, phenylethylsulfate, and hydroquinone sulfate. Thus, it appears that a sulfated two-ring steroid, such as 2-SMP, is also a substrate of SOAT. Because of its predominant expression in testis, we conclude that testes are also exposed to electrrophilic adduct-forming pyrene sulfates by uptake via SOAT. This uptake might even be related to the well-known risk of testicular cancer in tobacco smokers (37).

**Phylogenetic Relationship of SOAT, ASBT, and NTCP**—The evolutionary origin of the SLC10 transporter family was recently shown by a phylogenetic analysis of the SLC10A1–SLC10A6 genes from several mammalian and nonmammalian species (14). This analysis revealed two major clades of genes. Clade I comprises SLC10A1 (NTCP), SLC10A2 (ASBT), SLC10A4, and SLC10A6 (SOAT) genes; clade II contains SLC10A3 and SLC10A5 genes. Within clade I, SOAT is the sister group to ASBT, and SLC10A4 is the sister group to NTCP. This phylogenetic relationship explains the high sequence homology between SOAT and ASBT as well as the lower sequence homology between ASBT and NTCP. Functional transport properties of SLC10 carriers can overlap but might also be very divergent. A likely explanation would be that the common ancestor gene for SOAT, ASBT, and NTCP exerted transport of bile acids (either sulfoconjugated or non-sulfoconjugated) plus sulfoconjugated steroids but separated them during later subdivision into ASBT (only nonsulfoconjugated bile acids), SOAT (only sulfoconjugated bile acids), and NTCP (bile acids and sulfoconjugated bile acids, and sulfoconjugated steroids). At present, the varying local organ expression of these three SLC10 carriers combined with their individual substrate pattern reflects and causes broad physiological plasticity.

**Sodium Dependence of SOAT**—The driving force for the NTCP-mediated and ASBT-mediated transport of bile acids is provided by the inwardly directed Na\(^+\) gradient, which is maintained by the activity of the Na\(^+\)/K\(^+\)-ATPase in the plasma membrane as well as the negative intracellular potential. As
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demonstrated for rat Ntcp and human ASBT, these transporters perform an electrogenic transport cycle and move two Na\(^+\) ions for each bile acid molecule (9, 38–40). A comparable transport mechanism is also suggested for SOAT, because the transport of TLCS, DHEAS, E\(_1\)S, PREGS, 2-SMP, and 4-SMP by SOAT is also strictly sodium-dependent. Nonetheless, the cation selectivity of SOAT is not absolutely identical with that of ASBT. Whereas Li\(^+\) maintained about 40% of the SOAT transport function compared with Na\(^+\), Li\(^+\) is not accepted as a stimulating co-substrate of ASBT. On the other hand, equimolar substitutions of Na\(^+\) by choline abolished the transport function of both carriers (5, 9).

Membrane Expression and Topology of NTCP, ASBT, and SOAT—Hydrophobicity analyses of NTCP and ASBT proposed 7–9 TMDs, but experimental data strongly support a seven-TMD topology with an exoplasmic N terminus and a cytoplasmic C terminus (4, 8, 30, 41–43). For human SOAT, only one topology prediction program (TopPred II/KD-scale) supported this membrane topology, whereas most other calculations yielded eight transmembrane domains with an exoplasmic orientation of the N-terminal and C-terminal ends. In this paper, an N\(_{ex}\)/C\(_{cyt}\) trans-orientation was experimentally demonstrated, which is in accordance with the membrane topology of NTCP and ASBT but clearly eliminates a model with eight TMDs. Our experimental setup was not able to discriminate between a seven-TMD and nine-TMD membrane topology model. Nonetheless, based on the high sequence homology and almost identical hydrophobicity profiles of SOAT and ASBT, we suggest that SOAT displays a seven-TMD topology like ASBT.

SOAT Expression in Testis—We found that SOAT expression in testis is much higher compared with all other tissues. The physiological and/or pathophysiological relevance of SOAT expression in testis is unknown, but it could mean that the cellular import of the SOAT substrates DHEAS, E\(_1\)S, and PREGS would contribute to the overall androgen and estrogen production in this organ (44–46). Besides SOAT, other transporters for sulfoconjugated steroid hormones are expressed in the testes. These include the gonad-specific organic anion transporter, GST (also referred to as OATP6A1), and the organic solute carrier protein, OSCP1 (47, 48). However, no sodium dependence of these carriers was shown, and subcellular localization is poorly understood.

Transport of DHEAS in Human Placenta Trophoblasts—Besides testis, SOAT expression was also relatively high in human placenta. During pregnancy, this organ is the main source for hormone response of testis and placenta on sulfoconjugated steroid hormones and also for their toxicologic exposure to sulfoconjugated pyrene carcinogens as well as for placental transport of sulfoconjugated bile acids.

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