Na\textsuperscript{+}-d-glucose cotransporter in the kidney of Squalus acanthias: molecular identification and intrarenal distribution

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Althoff, Thorsten, Hartmut Hentschel, Jutta Luig, Hendrike Schütz, Myriam Kasch, and Rolf K.-H. Kinne. Na\textsuperscript{+}-d-glucose cotransporter in the kidney of Squalus acanthias: molecular identification and intrarenal distribution. Am J Physiol Regul Integr Comp Physiol 290: R1094–R1104, 2006. First published November 23, 2005; doi:10.1152/ajpregu.00334.2005.—Using primers against conserved regions of mammalian Na\textsuperscript{+}-d-glucose cotransporters (SGLT), a cDNA was cloned from the kidney of spiny dogfish shark (Squalus acanthias). On the basis of comparison of amino acid sequence, the SGLT from shark kidney has a high homology to the mammalian SGLT2. Computer analysis revealed that the elasmobranch protein is most similar to the mammalian proteins in the transmembrane regions and contains already all the amino acids identified to be functionally important, suggesting early conservation during evolution. Extramembranous loops show larger variations. This holds especially for loop 13, which has been implied as a phlorizin-binding domain. Antibodies were generated and the intrarenal distribution of the SGLT was studied in cryosections. In parallel, the nephron segments were identified by lectins. Positive immunoreactions were found in the proximal tubule in the early parts PIa and PIb and the late segment PIIb. The large PIIa segment of the proximal tubule showed no reaction. In contrast to the mammalian kidney also the late distal tubule, the collecting tubule, and the collecting duct showed immunoreactivity. The molecular information confirms previous vesicle studies in which a low affinity SGLT with a low stoichiometry has been observed and supports the notion of a similarity of the shark kidney SGLT to the mammalian SGLT2. Despite its presence in the late parts of the nephron, the absence of SGLT in the major part of the proximal tubule, the relatively low affinity, and in particular the low stoichiometry might explain the lack of a T\textsubscript{m} for d-glucose in the shark kidney.

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from Ambion. Integrity of the extracted RNA was checked by formaldehyde agarose gel electrophoresis (1.2% agarose). For first-strand synthesis in an Eppendorf Mastercycler Gradient from a total RNA template, we used oligo(dT)15 primer (Promega, Mannheim, Germany) and SuperScript II RT (GIBCO-BRL, Inchinman, UK). To prevent RNA contamination, all samples were treated with RNase H (GIBCO-BRL) after first-strand synthesis.

Preparation of cDNA library. cDNA libraries were prepared in plasmid pSPORT1 using a SuperScript kit for cDNA preparation from GIBCO-BRL following the supplier’s standard protocol. First strands were synthesized from total RNA on oligo(dT)15-Not I primer adapter and labeled with [α-32P]dCTP. After second-strand synthesis, Sal I adapters were ligated to the cDNA to introduce asymmetry, and Not I digestion exposed the 5′ extensions of Not I sites for ligation. cDNA was purified by size fractionation by column chromatography. The size-fractionated cDNA was then ligated into pSPORT1 plasmid vectors and transformed into Escherichia coli using electroporation.

Southern blot. Probes for dot-blot detection were labeled with DIG-DUTP by random priming using the DIG DNA labeling kit from Boehringer (Mannheim, Germany) according to the manufacturer’s protocol. For hybridization, we used the DIG Easy Hyb Granules from Boehringer. Probes were incubated at 68°C for 16 h. Thereafter, the blots were washed once with 2× saline-sodium-citrate (SSC) and 0.1% SDS for 5 min at room temperature, twice with 0.2× SSC and 0.1% SDS at 50°C for 15 min, and once with 0.2× SSC and 0.1% SDS at 60°C for 15 min. Hybridization at low stringency involved incubation at 40°C and the last two washings with 0.2× SSC at 50°C. Detection was performed with CSPD (Boehringer).

Cloning of SGLT from the kidney of Squalus acanthias. In a previous study on the mechanism of renal α-glucose transport in the early tetraodontiform fish (Oncorhyncus mykiss), it was found that the SGLT present in the brush-border membrane shared properties with both the SGLT1 and the SGLT2 cloned from mammalian tissues (9). Because of the evolutionary closeness and the combined functional properties, we chose trout mRNA to generate probes for the cloning of the transporters from the elasmobranch kidney. Based on the comparison of known slgt1 and slgt2 genes, we prepared several oligonucleotides from conserved regions and used them in RT-PCR (in all possible combinations) with trout kidney mRNA. Combination with the oligonucleotide sequences 5′- CCTGTTCTCGTATCACTTCAT-TCACCA-3′ and 5′-GCACTACCATCTAGAACTACATGG-3′ gave rise to a 550-bp fragment. Southern blot detection with a 32P-labeled 2,300 bp-probe from human SGLT2 was successful, and sequence analysis of the fragment revealed a high similarity with the mammalian slgt2 genes, so we named the probe slgt2k. Subsequently, the fragment was labeled and used to screen a library from trout kidney. A positive clone was identified and used to generate a probe of 1,573 bp. With the 1,573-bp fragment containing slgt2k probe, we screened the cDNA library from shark kidney. Positive clones were isolated and used for second-strand synthesis, restriction mapping, subcloning, and sequencing. In shark kidney, we found a 2,196-bp long cDNA. BLASTn search in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov; see Ref. 2) nucleotide database revealed high homology to SGLT for the cloned cDNA.

Plasmid preparation. Plasmids were isolated from E. coli using the Plasmid Kit from Qiagen (Hilden, Germany).

Sequencing. Sequencing was performed by labeling with BigDye terminator and reading on an ABI PRISM 3100 (Applied Biosystems) supplied by our institute.

Real-time PCR. Quantitative real-time PCR was performed in a GeneAmp 5700 Sequence Detection System from Applied Biosystems (Weiterstadt, Germany). All reagents used, including primers, probes, and disposables for real-time PCR, were also purchased from Applied Biosystems. The primers and TaqMan probe were designed using the Primer Express software from Applied Biosystems. The TaqMan probe was labeled with FAM-reporter and TAMRA quencher. We used the standard thermal protocol (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min), and reactions were performed in 50 μl containing 2× TaqMan Universal PCR Master Mix, 10 pmol TaqMan probe, 45 pmol forward and reverse primer, and 0–2 μl cDNA or plasmid solution as template. For each value, four replicates were used. The threshold of fluorescence signal was set to 0.06, and the baseline was set in a range from cycle 7 to 17, usually seven or eight cycles. As standards we used 7.50, 1.88, 0.47, 0.12, and 0.03 pg plasmid (141,000 copies/pg) cloned from shark SGLT2 to cover a range from cycle threshold (Ct) ~20 (7.50 pg) to 28 (0.03 pg). As template from the unknown samples, we used 20 ng cDNA.

For first analysis of the obtained real-time PCR data, we used the control software of the GeneAmp 5700, which calculated the quantity (in pg) of the cDNA of interest in each well from the measured Ct value according to the standard curve. In each run, the wells with the highest deviation to the other replicates were taken out. Further analysis was performed with Microsoft Excel. Finally, we calculated the number of copies from the quantity in picograms. All values are presented as the number of start copies in 20 ng cDNA with SE. Topology model. To generate a tentative topology model, we used TMHMM2.0 available online (www.cbs.dtu.dk). This program uses a hidden Markov model for the prediction of membrane-spanning regions in amino acid sequences (17, 25). We chose this program because according to Möller et al. (20) it “is currently the best performing transmembrane prediction program”. Possible sites for N-glycosylation, phosphorylation, etc., were determined using Motif Scan of Wisconsin Package 9.0 from Genetics Computer Group (Madison, Wisconsin) and PROSITE database (www.expasy.ch; see Ref. 7).

Uptake studies on Xenopus laevis oocytes. cRNA was transcribed from HindIII-linearized vector pSPORT1 using the T7 mMESSAGE mMACHINE Kit from Ambion according to the supplier’s protocol. Oocytes were injected with 30 ng cRNA coding for shark kidney SGLT in 30 nl water or 30 nl water as negative control on the same or one day after preparation, and transport was measured 3–5 days after injection. Until this, oocytes were stored in Barth’s solution supplemented with gentamycin under standard conditions. Before uptake, the oocytes were washed two times with Barth’s solution and subsequently preincubated in Na+−free uptake solution for 3 min. For Na+−dependent uptake, oocytes were placed in uptake solution [100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES-Tris (pH 7.5), 10 μM cytochalasin B, α-glucose in the appropriate concentration, 40 μM NaI or [14H]glucose; for Na+−independent uptake, NaCl was replaced with 100 mM N-methylglucamine. Uptake was performed for 20 min at room temperature, and then it was terminated by removing the uptake medium and washing three times with cold stop solution (same as solution for Na+-independent uptake). Each oocyte was placed in a single vial and lysed by addition of 10% SDS for 1 h. After addition of ReadyProtein+ scintillation solution (Beckman Coulter), counting was performed in a Beckman Coulter LS 6500. Experiments were performed two times with independent sets of 5–10 oocytes/tested condition. For analysis, data of the uptake experiments were pooled and analyzed with Microsoft Excel; Microcal Origin 6.0 was employed to calculate the nonlinear regression.

Generation of antibodies. For antibody production, loop 13 was chosen, and the respective peptide (SH-L13c) comprised of amino acids 551–644 was produced as glutathione S-transferase (GST) fusion protein. The corresponding DNA fragment was amplified from the cloned cDNA by PCR using special primers for the Gateway system from Invitrogen (Karlsruhe, Germany). These oligonucleotides consisted of a common part for recombination and the following sequence specific parts: 5′-GAC GAG AAA CAT CTG CAT CGC-3′ and 5′-GAT TTC TTC CAT TTT CCT CGC-3′. The PCR product was cloned directly into the pGEX-5 X 3 expression vector. In-frame cloning and correctness of the sequence were confirmed by DNA sequencing. GST fusion protein was expressed in S. cerevisiae.
BL21 E. coli cells and purified using glutathione-Sepharose according to the protocol provided by the supplier (Amersham Pharmacia Biotechnology). The purified protein was sent to Biotrend (Köln, Germany) for polyclonal antibody production in rabbits according to the standard protocol, and Biotrend purified the final anti-serum with protein A to obtain the IgG fraction.

Western blot. For Western blotting, tissue samples were homogenized in a HEPES-buffed sucrose solution [0.25 M sucrose, 10 mM HEPES (pH 7.4)-Tris, and protease inhibitor cocktail (for general use, Sigma)]. Membrane proteins were extracted by addition of 1% Triton X-100 [in 2.5 mM EDTA-Tris (pH 7.4) and 20 mM HEPES-Tris (pH 7.4)] and shaking for 1 h at 4°C. Insoluble material was pelleted in a centrifugation at 41,000 g at 4°C for 1 h. Protein concentration in the supernatant was determined by the method according to Lowry et al. (19) to ensure that equal amounts were loaded on the gel. Proteins were separated in 10% gels by SDS-PAGE according to Laemmli (18) and subsequently transferred to nitrocellulose membranes (Bio-Rad; see Ref. 26). To block unspecific binding sites, the membranes were incubated in 3% nonfat dry milk in PBS + 1% Tween 20 for at least 2 h. Proteins were detected with the respective primary antibody against shark kidney SGLT (1:3,000 in PBS + 1% Tween 20 + 2% milk; incubation at 4°C overnight) and a horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:2,000; Sigma) followed by detection with an enhanced chemiluminescence system from Perkin-Elmer (Dreieich, Germany). For antigen competition, 100 μg antigen were added to the antibodies and incubated for 1.5 h at room temperature before applying them on the membranes.

Immunohistochemistry. Tissue samples were prepared by dripping fixation fluid on kidney tissue exposed immediately after killing the animals by dissection in situ. Small pieces were cut from the posterior portion of the organ with constant drip of chilled fixative. The fixation fluid consisted of formaldehyde (4%, freshly prepared from paraformaldehyde) in PBS of ~900 mOsM/l (PBS, triple strength, to match the high osmolality of elasmobranch blood). The tissue was left in fresh fixative overnight at 4°C. After a rinse in PBS, the fixed pieces were embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Sections (5–7 μm) were obtained with a cryostat (Leica Microsystems, Bensheim, Germany). The sections were thawed on glass slides, briefly air-dried and stored at ~80°C, or immediately used for immunostaining. Before the histochemical reactions, skin milk (reconstituted from Carnation milk powder, 3% in PBS) was used for blocking unspecific immunoreactive binding sites. After being blocked for 1 h, the sections were incubated with specific primary antibodies (diluted 1:300 in PBS + 1% Tween 20) for another hour. For antigen competition, 25 μg antigen were added to the antibody solution before applying it to the slides. Alexa-Fluor 488 donkey anti-rabbit IgG or Alexa-Fluor 555 goat anti-rabbit IgG secondary antibodies (Molecular Probes, Eugene, OR) were diluted in the same way and bound to the primary antibody by a 1-h incubation at room temperature. Fluorescein-labeled lectins were applied as markers for specific renal structures. The lectins (as purchased from Vector Laboratories, Burlingame, CA) were as follows: Sophora japonica agglutinin (SJA), Griffonia simplicifolia lectin I (GSL I), Ricinus communis agglutinin I (RCA I), soybean agglutinin (SBA), Dolichos biflorus agglutinin (DBA), Ulex europaeus agglutinin I (UEA I), and concanavalin A. The lectins were diluted 1:150 in PBS and incubated for 20 min at room temperature. The DNA stain 4',6-diamidino-2-phenylindole was used to counterstain the nuclei. The stained sections were mounted with ProLong antifade solution (Molecular Probes) for multidimensional image acquisition.

RESULTS

Sequence of the cloned cDNA. The sequence of the cDNA cloned from the shark kidney is given in Fig. 1 and is submitted to EMBL-Bank as AM117565. The derived amino acid sequence was compared with those of already known mammalian Na+-glucose transporters. As depicted in Fig. 1 and Table 1 transmembrane regions, amino acids previously reported as functionally important and the glycosylation site are present in the shark transporter at almost identical sites as in the known transporters. Furthermore, Na+-solute symporter family signatures 1, 2, and 3 were detected. This suggests early conservation of these elements required for the transport activity. Differences were, however, found for putative phosphorylation sites, no protein kinase A (PKA) site is predicted but additional protein kinase C (PKC) sites (compared with hSGLT2). Major discrepancies are also found in the extramembraneous loops, in particular in loop 13. As shown in Fig. 2, the shark loop 13 is ~10 amino acids longer (typical for SGLT2s) and has a strikingly different sequence of amino acids. Figure 3 shows a dendrogram of alignment of amino acid sequences of the different SGLTs cloned thus far. It is evident that the identity is highest to the known SGLT2s (average 70.1%); the identity was significantly lower to SGLT1s (average 61.9%) and the lowest to SGLT3s. Work on the transporters in skate and hagfish, which in vesicle studies showed quite different properties, is in progress.

Expression of the gene in Xenopus laevis oocytes. From the cDNA of the cloned transporter cRNA was prepared and injected in oocytes, and the uptake of d-[3H]glucose was determined after 4 days of the expression period. As shown in Fig. 4A, oocytes injected with shark cDNA-derived cRNA showed significant (P < 0.07) Na+-dependent uptake of d-glucose. In water-injected oocytes, there was no significant difference in uptake in the presence or absence of Na+. As shown in Fig. 4B, the total uptake in cRNA-injected oocytes was composed of two components. Uptake in the absence of Na+ increased in a linear fashion and was unsaturatable (at least in the investigated range up to 10 μM). The Na+-dependent, SGLT-mediated uptake clearly fitted to a saturation curve. The apparent K_m of this Na+-dependent transport activity was 0.57 mmol/l.

Tissue distribution. In mammals, SGLT2 is restricted to the kidney, whereas SGLT1 is present in kidney and in small intestine. To investigate the expression of shark SGLT in various tissues, we used quantitative real-time PCR with TaqMan probes. Here we found that shark kidney contained 29,700 ± 13,100 (n = 4) copies/20 ng cDNA, whereas in intestine there were only 3,300 ± 1,900 (n = 4) copies.

Immunohistochemistry. From the amino acid sequence of loop 13, an epitope was selected to generate epitope-specific antibodies. As shown in Fig. 5, the antibody recognized mainly a protein of the expected molecular weight of 74,700 in Western blots of the kidney homogenate. The presence of the second band is probably because of a difference in glycosylation related to the intrarenal distribution (see below). Reactions could be abolished when the antibody was preincubated with the antigen. Similarly, the signal was absent when preimmune serum was used.

Identification of renal structures. The complex renal architecture of Squalus acanthias, and other marine elasmobranch
fish, involves a distinct zonation of the tissue (for review, see e.g., Refs. 5, 11, and 13). Thus cross sections through the excretory opisthonephric kidney generally reveal the following three characteristic regions: 1) the zone of lateral countercur-rent bundles, 2) the mesial tissue zone, and 3) a region between the two zones, where glomeruli abound. A schematic drawing of the anatomic organization of a single dogfish nephron is shown in Fig. 6. As the renal tubule takes a regular course alternating through the zones, it performs two hairpin loops in the bundles and two extended loops with multiple bends in the mesial tissue, before it joins the collecting tubule-collecting duct system. Figure 6 also summarizes the immunostaining results described in detail below.

**Localization of SGLT2 protein in the renal tissue.** As shown in Fig. 7A, anti-SGLT antiserum labeled membranes of renal tubule cells of various distinct nephron subsegments and epithelial cells of the collecting tubule-collecting duct system. By contrast, glomeruli displayed no immunoreactivity. For each of the nephron segments described below, where labeling by immune anti-SGLT antiserum was present, no fluorescence signal could be observed after preincubation of the antibody with the antigen (Fig. 7B) or after exposure to preimmune serum (Fig. 7C).

**Glomerulus.** To clearly distinguish the different nephron segments, counterstaining with lectins was performed (also see Refs. 10, 12, and 14). The glomeruli are located in a narrow region between the two renal tissue zones, namely the mesial tissue and the lateral bundle zone. The latter is present as a superficial layer of ~1 mm thickness on the kidney tissue. On histological cross sections of dogfish kidney, the lateral bundles can be seen frequently extending in the center of a section, as the renal surface follows the contours of two adjacent kidney lobules.

In our preparations, glomeruli were distinctly stained by GSL I fluorochrome (mesangial region) and soybean agglutinin SBA, lining glomerular capillaries at the presumed glomerular basement membrane. With the anti-SGLT antibodies, no reaction was observed (Fig. 8A).

**Neck segment.** The neck segment (NS) originates at the parietal layer of Bowman’s capsule and extends deep in the lateral bundle. Thus the NS is a tubular profile in many cross sections through the bundles. The NS contains abundant multiciliary cells, especially in the initial region near the glomerulus. The multiciliary cells are endowed with flames of cilia, which reacted with anti-tubulin antibody (Fig. 7). The majority of the lectins did not bind to this segment. Weak staining...
occurred after incubation with soybean agglutinin. Anti-SGLT antibodies did not bind to the NS (Fig. 8A).

**Proximal tubule segment PI.** The proximal tubule of elasmobranch fish runs through all regions of the kidney. It originates in the lateral bundle (proximal tubule PIa) with low prismatic epithelial cells. This initial portion of the proximal tubule reacted weakly with lectins GSL I and PNA and moderately with SBA. Anti-SGLT antibodies bound weakly at the apical brush border at the luminal side of the epithelium (Fig. 8B). The subsequent proximal tubule PIb is located in the vicinity of the glomeruli. It showed strong reaction with lectins RCA-I, SBA, and PNA and weak reaction with GSL I. The apical region of the epithelial cells and the brush border was distinctly reactive with anti-SGLT antibodies. The reaction frequently was very strong, and the signal obtained by fluorescence microscopy was the highest compared with other antibody-binding renal structures (Fig. 8C).

**Proximal tubule segment PII.** The proximal tubule segment PII is exclusively located in the mesial tissue. In *Squalus* it consists of two subsequent portions. Large tubular profiles of PIIa followed by the small tubular profiles of PIIb. PIIa reacted weakly with lectins GSL I and RCA I and only faintly with SBA. In contrast to the other proximal tubule segments, anti-SGLT antibodies failed to bind to the segment PIIa (Fig. 8D). In contrast to the large tubular profiles of PIIa, the small profiles of the proximal tubule segment PIIb reacted with many lectins (GSL I, RCA I, SBA, DBA, and PNA). With the anti-SGLT antibody, the apical cell membrane (brush border) showed distinct to strong reactions (Fig. 8D).

**Intermediate segment.** The connection between the proximal tubule and the distal tubule is made by a segment that is morphologically very similar to the neck segment. The intermediate segment (IS) is located in the vicinity of the glomeruli.

### Table 1. Comparison of conserved amino acids from SGLTs

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<tr>
<th>Function</th>
<th>SGLT1</th>
<th>Shark SGLT</th>
<th>SGLT2</th>
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<tr>
<td>Sugar binding</td>
<td>Gln&lt;sup&gt;457&lt;/sup&gt; (4)</td>
<td>Gln&lt;sup&gt;456&lt;/sup&gt;</td>
<td>Gln&lt;sup&gt;457&lt;/sup&gt; (4)</td>
</tr>
<tr>
<td>Sodium binding</td>
<td>Lys&lt;sup&gt;231&lt;/sup&gt; (23)</td>
<td>Lys&lt;sup&gt;320&lt;/sup&gt;</td>
<td>Lys&lt;sup&gt;321&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phlorizin binding</td>
<td>Cys&lt;sup&gt;608&lt;/sup&gt; (15)</td>
<td>Cys&lt;sup&gt;619&lt;/sup&gt;</td>
<td>Cys&lt;sup&gt;615&lt;/sup&gt;</td>
</tr>
<tr>
<td>Conserved amino acids</td>
<td>Gly&lt;sup&gt;595&lt;/sup&gt; (27)</td>
<td>Gly&lt;sup&gt;595&lt;/sup&gt;</td>
<td>Gly&lt;sup&gt;597&lt;/sup&gt;</td>
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<tr>
<td>N-glycosylation</td>
<td>Ser&lt;sup&gt;562&lt;/sup&gt; (23)</td>
<td>Ser&lt;sup&gt;561&lt;/sup&gt;</td>
<td>Ser&lt;sup&gt;562&lt;/sup&gt;</td>
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<tr>
<td>Phosphorylation (PKC)</td>
<td>Thr&lt;sup&gt;580&lt;/sup&gt; (31)</td>
<td>Thr&lt;sup&gt;48&lt;/sup&gt;</td>
<td>Thr&lt;sup&gt;47&lt;/sup&gt;</td>
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<tr>
<td>Ser&lt;sup&gt;303&lt;/sup&gt; (31)</td>
<td>Ser&lt;sup&gt;418&lt;/sup&gt; (31)</td>
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<td>Ser&lt;sup&gt;414&lt;/sup&gt; (Ser&lt;sup&gt;411&lt;/sup&gt;)</td>
<td>Ser&lt;sup&gt;414&lt;/sup&gt; (Ser&lt;sup&gt;411&lt;/sup&gt;)</td>
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SGLT, Na<sup>+</sup>-D-glucose cotransporter; PKC, protein kinase C; PKA, protein kinase A. Numbers in parentheses are the corresponding references.

Fig. 2. Comparison of amino acid sequences of shark kidney (kid) SGLT (saSGLT) loop 13 with human intestine (int) SGLT1 and human kidney SGLT2 (hSGLT) loop 13. Amino acids that appear only in shark are underlined. Predicted transmembrane regions are shaded gray, whereas putative functionally important amino acids are shaded black.

Fig. 3. Dendrogram of alignment of amino acid sequences of different SGLTs. Amino acid sequences of known SGLTs were aligned using GCG. Values were calculated by aligning each transporter with the shark transporter. Accession Nos. of nucleotide sequences: human intestine SGLT1 M24847; human kidney SGLT2 M95549; human SGLT3 NM_014227 (same as AJ133127); rat intestine SGLT1 D16101 (same as U03120); rat kidney SGLT2 U29881; mouse SGLT1 NM_019810; mouse kidney SGLT2 NM_133254; mouse SGLT3 AF411960; rabbit SGLT1 X55355; pig SGLT3 P31636 (same as L02900); sheep intestine SGLT1 X82411 (same as X82410); dog SGLT1 AY772536; dog SGLT2 AY772537; horse intestine SGLT1 AY292081; cow mammary gland SGLT1 AF508807; cow kidney SGLT2 AY208941. Genome sequencing-derived sequences and partial sequences were not considered. Transporters whose transport characteristics have been shown are underlined.
and extends in the lateral bundle. The IS contains multiciliary cells like the other segments of the proximal nephron. The IS was stained by lectin fluorochromes SJA, GSL I, SBA, and DBA and (faintly) with UEA I. In our experiments, SJA specifically labeled only this segment. Immunoreactivity to the anti-SGLT antibodies was not present (Fig. 9A).

Early distal tubule. The early distal tubule (EDT) segment is exclusively present in the lateral bundles. Lectins GSL I and SBA markedly bound to this segment. Weak reaction was observed with DBA and faint reaction with UEA I. With anti-SGLT antibodies, only faint binding was observed at the apical membrane (Fig. 9B).

Late distal tubule. As the early distal tubule tapers at its end it gradually becomes the late distal tubule (LDT) segment, passing through the region of the glomeruli and running between the loops of the segments PIa and PIb in the mesial tissue. The LDT was well marked by binding of lectins UEA I, GSL I, and SBA and showed faint reaction with DBA. Sur-
prisingly, the apical region of the late distal tubule exhibited strong reaction with anti-SGLT antibodies. A speckled staining pattern beneath the apical cell membrane suggests in addition the presence of the transporter in small apical vesicles (Fig. 9C).

Collecting tubule-collecting duct system. In the mesial tissue, the late distal tubule gradually becomes the collecting tubule with no distinct borderline between these two tubular segments. The collecting tubule thereafter passes the region of the glomeruli and delves into the lateral bundle where it joins the collecting duct. The lectins GSL I, RCA I, SBA, and UEA I bound to the collecting tubule. Again, the apical regions of epithelial cells of the collecting tubule and the collecting ducts were strongly reactive with anti-SGLT antibodies (Fig. 9D).

All of the above findings are compiled in Table 2, which summarizes intrarenal locations and the intensity of the immunoreactivity observed.

DISCUSSION

So far, SGLTs have been cloned and characterized mainly from mammalian species like rodents and humans. Here we report the cloning and characterization of an SGLT from the ancient vertebrate Squalus acanthias.

Screening a library from shark kidney mRNA, we isolated a cDNA that showed sufficient homology to SGLTs. The derived amino acid sequence showed further characteristics of SGLT. The SGLT from shark has 14 transmembrane helices, which is, according to Turk and Wright (27), typical for many eukaryotic transporters and especially for Na$^+$-dependent D-glucose transporters. Furthermore, we also found some typical putative functional elements (binding sites, phosphorylation sites, etc.) and other amino acids with still unknown function that are well conserved throughout all SGLTs. The highest conservation is thereby observed within the transmembrane regions and small linking loops. In detail, these are for sugar-binding Gln[457] [both in SGLT1 (4) and SGLT2] which corresponds to Gln[456] in shark. Díez-Sampedro et al. (4) also identified Thr[460] for sugar binding in SGLT1, which is replaced by Ser[460] in shark. Díez-Sampedro et al. (4) also identified Thr[460] for sugar binding in SGLT1, which is replaced by Ser[460] in SGLT2. In the SGLT from shark, there is a Thr at position 459 and a Ser at position 460. All of these residues are located in transmembrane helix 11 that is part of the sugar translocation pathway through the membrane (22). In the extracellular loop 8 Kasch (15) described Cys[351] and Cys[361] [SGLT1 (15) and SGLT2] as involved in defining sugar affinity of the transporter that corresponds to Cys[350] and Cys[360] in shark. For Na$^+$ binding in SGLT1, Panayotova-Heiermann et al. (23) postu-
lated Lys321 in transmembrane helix 8, which can also be found in SGLT2. In shark this is Lys320. Novakova et al. (21) also found out that the region around Cys608 and this residue itself in SGLT1 (Cys615 in SGLT2) are involved in the binding of the inhibitor phlorizin. Therefore, the presence of Na+/H-solute symporter signatures, the overall identities, and especially those at the functional residues clearly define the new transporter as SGLT.

More diversity can be found at the NH2 terminus, and within the larger loops, especially loop 13, which differs in length, amino acid composition, and sequence from that in the other SGLTs. For example, there are two cysteines at positions 614 and 615 in shark SGLT, whereas mammals have only one. Recent studies on the phlorizin-binding properties of isolated loop 13 from the rabbit SGLT1 showed that a disulfide bridge is formed between Cys560 and Cys608 (33). A probably corresponding cysteine is located at 546 in the shark (and similarly in human kidney SGLT2). Because of the larger distance between the two cysteines in the shark, a different folding of loop 13 might occur. This fact might explain the different inhibition pattern for phlorizin observed in transport studies with shark kidney brush-border membrane vesicles compared with rabbit brush-border membrane (16).

As already mentioned above, there are several putative phosphorylation sites in the transporter. The positions of the sites are similar to those found by Wright et al. (31), in loops 1, 9, and 13 of human SGLT1. In shark kidney SGLT, half of the phosphorylation sites are for CK2 and half for PKC, but none for a PKA. Although the regulation of SGLTs by phosphorylation might not be directly correlated with the presence of phosphorylation consensus sequences (31), these may be a hint for different regulative mechanisms of SGLT in shark. This point remains to be investigated further.

Transport studies on Xenopus laevis oocytes injected with cRNA coding for shark kidney SGLT showed an apparent

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Fig. 8. Tissue cross sections from shark kidney. Triple staining with lectin fluorescein conjugate (green), anti-SGLT primary antibody and Alexa fluor 555 anti-rabbit IgG conjugate (red), and nuclear counterstain DAPI (blue). A: section through GL and NS. Anti-SGLT antibody does not bind at GL and NS, whereas *Griffonia simplicifolia* lectin I (GSL-I) does. B: cross section through a lateral countercurrent bundle. Pla is stained with peanut agglutinin (PNA) and reacts with anti-SGLT antibody. C: section through Plb in the vicinity of GL. SGLT protein is present at the apical brush-border region of epithelial cells. Containing with soybean agglutinin (SBA). D: section through Plia and Plb in the MT. Signal for anti-SGLT antibody is absent in Plia and markedly present in Plb. GSL-I stains both.
higher affinity (0.57 mM) than that found in the previous vesicle studies (1.9 mM; see Ref. 16) and is more in the range observed for mammalian SGLT1 transporters. However, affinities can vary drastically in heterologous expression systems when differences in the driving forces exist or different experimental setups are employed. The vesicle studies showed, however, clearly that the stoichiometry of the Na\(^+\)/H\(^+\)-D-glucose cotransport was 1 Na\(^+\) to 1 D-glucose, the hallmark property of the SGLT2s. The sequence comparison and the dendrogram also indicate that the SGLT expressed in shark kidney belongs to the SGLT2 family. Furthermore, the tissue expression of shark SGLT is more similar to mammalian tissues in that SGLT2 is highly expressed in the kidney, but almost absent in the intestine. The absence of expression in the shark intestine...
has been confirmed by immunostaining of the tissue with the anti-shark SGLT antibodies used in this study (24).

The quite weak expression of renal SGLT2 in the shark intestine leads to the question of which transporter serves for the absorption of d-glucose in this organ. The fact that we could not detect significant expression does not mean that there is no SGLT at all. Transport studies on brush-border membrane vesicles isolated from shark intestine revealed a Na+-dependent d-glucose uptake mechanism with an apparent $K_m$ of 0.027 mmol/l and a high affinity for d-galactose (1). These are functional properties of SGLT1. Thus, in shark intestine, similar to mammals, probably a yet unidentified SGLT1 transporter mediates d-glucose absorption.

Interestingly, the intrarenal distribution in the shark kidney differs from that found in the mammalian kidney. Typically, SGLT2 is found in the early part of the proximal tubule and is involved in the bulk reabsorption of d-glucose at high intraluminal sugar concentrations, whereas the later parts express SGLT1, the high-affinity system capable to operate against higher concentration gradients and at lower intraluminal glucose concentrations (28). In the shark, the early part of the proximal tubule also expresses a SGLT2-like transporter. The major and most abundant segment of the proximal tubule, PIIa, shows, however, no staining with the anti-SGLT antibodies. This segment has mainly secretory function in the shark kidney. Thus, in the elasmobranch, the separation between absorptive and secretory functions seems to be more pronounced than in the mammalian tubule. Indeed, the same seems to hold for the aglomerular teleost *Opsanus tau* where isolated brush-border membranes from the proximal tubule were shown to exhibit taurine transport, involved in taurine secretion, but no Na+-dependent d-glucose transport (29).

The presence of SGLT in the very late parts of the nephron, namely the collecting tubule-collecting duct system, is very interesting, since this is in contrast to mammalian systems. In mammals, the reabsorption of sugars, amino acids, and ions takes place predominantly in the proximal tubule, whereas the late parts serve for concentrating the urine by reabsorbing urea and water. One possible explanation for the presence of SGLT in the collecting tubule of shark kidney could be that, because of the lack of the transporter in PIIa, glucose is not removed as efficiently as in the mammalian SGLT2/SGLT1 system, and the transporter has to be expressed at a considerably longer distance along the tubule.

The expression in the later tubule segments can also be regarded as a result of the attempt to recover as much d-glucose from the primary urine as possible, since the shark diet (mainly squids) is low in carbohydrates. However, the success of this attempt seems to be limited. Studies by Boylan and Antkowiak (3) have shown that even at high blood glucose concentrations no saturation of the renal transport ($T_m$) can be observed. This could be because of the relatively low affinity and, in particular, the low stoichiometry of the transporter and thus the inability of the epithelium to generate steep d-glucose gradients in the late distal tubule.

Another possibility could be that, in the collecting tubule/collecting duct of shark kidney, SGLT has a different function than glucose transport. For example, SGLTs can also transport urea and water (32). Thus here SGLT could mediate the transport of water and/or urea. This hypothesis remains to be investigated further.

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