Rat renal glucose transporter SGLT1 exhibits zonal distribution and androgen-dependent gender differences

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The efficient reabsorption of the filtered hexoses in the mammalian proximal tubule is achieved by two Na+-dependent glucose cotransporters (SGLT1 and SGLT2), localized in the brush-border membranes (BBM) of proximal tubule cells (Refs. 11 and 34 and references therein). The two major brush-border glucose transporters in the proximal tubule, low-affinity/high-capacity SGLT2 and high-affinity/low-capacity SGLT1, differ in their affinity for glucose and Na+ (SGLT1 > SGLT2), their sensitivity to inhibitory phlorizin (SGLT1 < SGLT2), and their sugar selectivity (SGLT1 transports glucose and galactose equally well, whereas SGLT2 transports glucose at least 10 times better). At the protein level, both transporters have ~660 amino acid residues that share 59% homology and probably function as glycosylated monomers (Refs. 11, 33, and 34, and references therein), which on Western blots appear as a broad, 70- to 80-kDa protein band, as indicated by recent findings for SGLT1 in the rat intestinal brush-border membrane (BBM) and outer stripe (OS) of the renal proximal tubule (S3) (8, 9).

Based on transport studies in isolated, microperfused proximal tubule segments (3), transport and stoichiometric studies in isolated BBM vesicles from the outer cortex and outer medulla of rabbit kidney (28–30), and Northern blot and in situ hybridization studies of the specific mRNAs in rabbit and rat kidney tissues (15, 21, 35), the expression of SGLT1 and SGLT2 exhibits a distinct segmental distribution; SGLT2 is localized to the proximal tubule S1 and S2 segments in the cortex, whereas SGLT1 is localized to the proximal tubule S3 segments in the outer stripe and medullary rays. Such a distribution ensures SGLT2-mediated reabsorption of the bulk of filtered sugar along the S1 and S2 segments, and SGLT1-mediated reabsorption of the remaining sugar in the S3 segment. However, the exact localization of SGLT1 (and SGLT2) and its expression level along the renal nephron are not entirely clear because good and specific antibodies for these proteins have not been generated thus far. The study by Takata et al. (26) identified SGLT1 as a 77-kDa protein that was localized in BBM along the entire proximal tubule in rats, which was somehow at odds with the predominant finding of SGLT1-specific mRNA by Northern blotting and in situ hybridization in the S3 segments (15, 21, 35). However, the study by Pajor et al. (20), using anti-peptide antibodies against rabbit SGLT1, demonstrated that the protein was more abundant in the outer medulla than the outer cortex of the rabbit kidney.

We have recently raised a polyclonal antibody against the specific peptide deduced from the amino acid sequence of rat kidney SGLT1 (rSGLT1) (8, 9). The affinity-purified sample of this antibody labeled a 70- to 80-kDa protein band on immunoblots of small intestinal BBM and immunocytochemically stained the apical domain of the rat small intestine and endothelial cells in capillaries of the rat skeletal and heart muscle and rat and pig brains (8, 9). Here, we used the same antibody to characterize and immunolocalize rSGLT1 in the rat kidney. Our data confirm a predominant localization of the rSGLT1-related ~75-kDa protein in proximal tubule S3 segments and reveal gender differences in the expression of functional

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SGLT1 protein and its mRNA, which are caused by inhibitory action of androgens.

MATERIALS AND METHODS

Animals and treatment. Adult (10–12 wk old) and prepubertal (25 days old) male and female Wistar strain rats were from the breeding colony at the Institute in Zagreb, whereas adult (10–12 wk old) C57BL/6 male mice were purchased from the breeding colony at the Department of Physiology, School of Medicine, University of Zagreb. Animals were bred and maintained according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Before and during experiments, animals had free access to standard pelleted food and tap water. The studies were approved by the Institutional Ethics Committee.

A gonadectomy was performed in the adult animals. Male rats were castrated by a scrotal route, whereas female rats were ovarioctomized by the dorsal (lumbar) approach under proper anesthesia (Narketan, 80 mg/kg body mass/Xylapan, 12 mg/kg body mass ip). The sham-operated animals underwent the same procedure, except the respective organs were not removed. Eight days later, animals underwent a subcutaneous treatment with either testosterone enantate or estradiol dipropionate, or progesterone at a dose of (each) 2.5 mg·kg body mass\(^{-1}\)·day\(^{-1}\) for 8 days. The hormones were injected as a sunflower oil solution. Control rats were treated with an equivalent amount of sunflower oil (0.5 mg·kg body mass\(^{-1}\)·day\(^{-1}\) for 8 days). As recently reported by us, these operational and hormonal treatments in rats caused distinct patterns of expression of organic anion transporters OAT1 and OAT3 in the renal proximal tubules (17). Mice were used intact.

Antibodies and other materials. Polyclonal immune serum against the peptide specific for rat SGLT1 (rSGLT1; amino acids 582–600: EEDPKDTEIDAEAPQKEK) was raised in rabbits (8, 9). The specific antibody (rSGLT1-Ab) was affinity purified from the immune serum via the antigenic peptide-coupled support (9). The use of a polyclonal anti-Na-K-ATPase \(\alpha\)-subunit antibody has been described (2, 6). A monoclonal antibody against \(\alpha\)-actin was purchased commercially (Chemicon, Temecula, CA).

In a set of experiments, we have immunoadsorbed an anti-40-kDa protein antibody (40 kDa-Ab) from the immune serum by using Immoblot strips, with steps described in detail previously (23). In preliminary experiments, this antibody was tested, properly diluted with PBS, and then used in immunocytochemical and immunoblotting studies with rat and mouse tissue sections and membrane preparations. The conditions used with this antibody completely matched those used with rSGLT1-Ab.

Secondary antibodies were purchased commercially from Jackson ImmunoResearch Laboratories (West Grove, PA) or Kirkegaard and Perry (Gaithersburg, MD) and included the CY3-labeled (GARCY3) or alkaline phosphatase-labeled (GARAP) goat anti-rabbit IgG or FITC-labeled (GMF) or alkaline phosphatase-labeled (GAMAP) goat anti-mouse IgG.

Anesthetics (Narketan and Xylapan) were purchased from Chassot (Bern, Switzerland). Oil solutions of testosterone enanthate, estradiol dipropionate, and progesterone were from RotexMedica (Trittau, Germany), Galenika (Zemun, Yugoslavia), and Prolék (Belgrade, Yugoslavia), respectively. The molecular weight standards, used in immunoblotting, were either from Gibco BRL/Invitrogen (Carlsbad, CA) or from Bio-Rad (Hercules, CA). \(\alpha\)-[\(\text{H}\)]-galactose (specific activity 40–60 Ci/mmol) was purchased from BioTrend Chemicalien, TechnologieZentrum (Cologne, Germany). The RNA-stabilizing reagent RNAlater, and other chemicals and specific columns for isolation of total RNA and mRNA from the renal tissues, were from Qiagen (Qiagen, Hilden, Germany).

Various other chemicals (cold D-galactose, phlorizin, valinomycin, etc.) were the highest purity available and were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Tissue fixation and immunocytochemistry. In anesthetized animals, the circulatory system was perfused via the left ventricle of the heart, using a Masterflex pump (Cole-Parmer, Chicago, IL), first with aerated (95% \(\text{O}_2\)-5% \(\text{CO}_2\)) and temperature-equilibrated (37°C) PBS (in mM: 137 NaCl, 2.7 KCl, 8 Na\(_2\)HPO\(_4\), 2 K\(_2\)PO\(_4\), pH 7.4) for 2–3 min to remove blood and then with 10 (mice) or 150 (mL rats) fixative (4% paraformaldehyde in PBS) for 4–5 min. The kidneys, small intestine (jejunum), uterus, and abdominal aorta were removed, sliced, and kept overnight in the same fixative at 4°C, followed by extensive washing in PBS and storage in PBS containing 0.02% Na\(_3\)EDTA at 4°C until further use.

To cut 4-μm frozen sections, tissue slices were infiltrated with 30% sucrose (in PBS) overnight, embedded in OCT medium (Tissue-Tek, Sakura, Japan), frozen at –25°C, and sectioned with a Leica CM 1850 cryostat (Leica Instruments, Nussloch, Germany). Sections were collected on Superfrost/Plus Microscope slides (Fischer Scientific), dried at room temperature for 2–3 h, and kept refrigerated until further use.

Before being stained with the antibody, sections were rehydrated in PBS for 15 min, heated in 10 mM citrate buffer, pH 6, for 20 min (4 cycles, 5 min each at 800 W) in a microwave oven, followed by cooling down to room temperature in the same buffer for 20 min.

Subsequently, sections were incubated in a wet chamber with 0.5% Triton X-100 (in PBS) for 5 min (to maximally expose antibody binding sites), rinsed with PBS (5 X 5 min each), incubated for 20 min with BSA (1% BSA in PBS) to block the nonspecific antibody binding, incubated with the rSGLT1-Ab (diluted 1:50 with PBS) or 40 kDa-Ab overnight in a refrigerator, rinsed with PBS (4 × 5 min each), incubated with GARCY3 (1.6 μg/mL in PBS) at room temperature for 60 min, rinsed with PBS (4 × 5 min each), and mounted in a fluorescence fading retardant (Vectashield, Vector Laboratories, Burlingame, CA).

To test the staining specificity, the rSGLT1-Ab was blocked with the immunizing peptide (final concentration of the peptide: 0.5 mg/mL) for 4 h at room temperature before use in the above-described immunofluorescence assay.

To perform double staining (rSGLT1-Ab+actin), sections were first incubated with the rSGLT1-Ab (1:50) overnight, rinsed with PBS, then incubated with the monoclonal anti-actin antibody (5 μg/mL in PBS) for 2 h at room temperature, rinsed with PBS, and incubated with GARCY3 at room temperature for 1 h. After being rinsed with PBS, sections were incubated with GMF (15 μg/mL in PBS) at room temperature for 1 h, rinsed with PBS, covered with Vectashield, and prepared for microscopy.

The stained sections were examined and photographed with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) using a Spot RT Slider camera and software (Diagnostic Instruments, Sterling Heights, MI). The photos were imported into Adobe Photoshop 6.0 for processing and labeling.

Preparation of tissue homogenates and membranes. The animals were killed by decapitation. The kidneys were removed and sagittally sliced. The cortex and outer stripe were dissected manually and used as separate tissue pools, whereas the inner stripe and inner medulla were processed as a single sample. The kidneys from one mouse were used in toto (without separation of various zones). The abdominal aortas, collected from five male rats, and uteri, collected from three females, were processed as separate tissue pools. The respective tissues were homogenized (10% homogenate) in a chilled buffer (in mM: 300 mannitol, 5 EGTA, 12 Tris-HCl, pH 7.4, 1 PMSF, and 0.1 benzamidine as well as 0.1 μg/ml antipain) with a Powergen 125 homogenizer (Fisher Scientific) at the maximal setting (1-min homogenization, 1-min pause, 1-min homogenization). The total cell membranes were isolated from these homogenates by first removing cell debris by centrifugation in a refrigerated high-speed centrifuge (Sorvall RC-5C, rotor SS34, Sorvall Instruments, Newtown, CT) at 6,000 g for 15 min. The pellets were discarded, and the supernatants were then centrifuged at 150,000 g for 1 h (ultracentrifuge Sorvall OTD-
Combi, rotor T-875). The final pellets (total cell membranes) were resuspended in homogenizing buffer.

BLM were isolated from the homogenates of various tissue zones by Percoll density gradient centrifugation (24), whereas BBM were isolated from the homogenates of cortical and outer stripe tissues of the rat kidney, or from the whole mouse kidney, by Mg2+/EGTA precipitation (4). The intestinal BBM were isolated from the rat jejunum, using the same method as for the renal membranes. As shown in our previous publications (19, 23), BBM isolated with this method are enriched 10- to 15-fold in the activity of marker enzyme leucine arylamidase, with minimal contamination of BLM and other intracellular membranes, whereas the data in Fig. 5 indicate that the isolated BLM preparations from various kidney zones were strongly enriched in the 100-kDa protein band corresponding to the Na+/K+-ATPase α-subunit, with minimal contribution by the BBM, as judged from the weak or negative BBM-specific, SGLT1-related 75-kDa protein band. After dispersal of membranes in an appropriate volume of buffer (150 mM mannitol, 2.5 mM EGTA, 6 mM HEPES/Tris, pH 7.4) and measurement of proteins by the Bradford assay (5), the membrane preparations were stored at −70°C until further use for immunoblotting studies.

**SDS-PAGE and Western blotting.** The membrane samples were thawed at 37°C, mixed with sample buffer, which contained 1% SDS, 12% vol/vol glycerol, 30 mM Tris-HCl, pH 6.8, without (for SGLT1 and 40 kDa-protein) or with (for Na-K-ATPase and actin) 5% β-mercaptoethanol (β-ME). Samples were denatured at either 65°C for 15 min (for SGLT1 and 40 kDa-protein) or 95°C for 5 min (for Na-K-ATPase and actin). Proteins were separated through 10% SDS-PAGE minigels using the Vertical Gel Electrophoresis System and then electrophoretically wet-transferred using a Mini-Trans-Blot Electrophoretic Transfer Cell (both Bio-Rad) to an Immobilon membrane (Millipore, Bedford, MA). Following transfer, the Immobilon membrane was briefly stained with Coomassie brilliant blue to check for the efficiency of the transfer, destained, blocked in blotting buffer (5% nonfat dry milk, 0.15 M NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.4), and incubated at 4°C overnight (12-14 h) in the same buffer containing either the rSGLT1-Ab (1:500), 40 kDa-Ab (1:500), anti-Na-K-ATPase antibody (1:1,000), or anti-actin antibody (0.5 μg/ml). Then, the membrane was washed four times for 15 min with blotting buffer, incubated for 60 min in the same buffer that contained either 0.1 μg/ml GARAP or 0.5 μg/ml GAMAP, washed again, and stained for alkaline phosphatase activity using the 5-bromo-4-chloro-3-indolyl phosphate (1.65 mg/ml)/nitro blue tetrazolium (3.3 mg/ml), in 20 mM Tris-HCl buffer (pH 9.0), method as an indicator. To demonstrate specific labeling, the rSGLT1-Ab and 40 kDa-Ab were preincubated with the immunizing peptide (final concentration of the peptide: 0.5 mg/ml) for 4 h at room temperature and then used in an immunoblotting assay as described above.

The Western blot studies were performed with isolated BBM, BLM, or, in some experiments, with total cell membranes. The amount of protein/lane was 50–60 μg for BBM, 10–60 μg for BLM, and 50–80 μg for total cell membranes and is indicated in the respective figure legends. In preliminary experiments, we showed that the data obtained with purified specific membranes matched those with total cell membranes. The amount of the peptide: 0.5 mg/ml) for 4 h at room temperature and then used in an immunoblotting assay as described above.

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rSGLT1-Ab labeled two major bands, at ~40 and ~75 kDa (−P), which were abolished after preincubation of the antibody with the immunizing peptide (+P). In tissue cryosections (Fig. 2), the antibody brightly stained the absorptive cell BBM in the villi and intestinal glands and weakly the smooth muscles in submucosa and muscularis externa (Fig. 2, A and C). This staining was abolished after the antibody was blocked with the immunizing peptide (Fig. 2D). These experiments, therefore, confirmed the previously reported specific labeling of the 70- to 80-kDa protein band in the jejunal BBM and staining of the jejunal apical domain with the same antibody in rats (8, 9). However, our data revealed that the antibody also labels a previously unrecognized 40-kDa protein band and stains smooth muscles.

rSGLT1-Ab-related staining along the rat nephron. Similar to the findings in the jejunal BBM, the rSGLT1-Ab labeled the same, peptide-blockable compact 40-kDa and more complex 75-kDa protein bands in BBM isolated from the rat kidney outer stripe (Fig. 1). The 75-kDa band consisted of a strong middle band and two to three weaker side bands, possibly indicating different glycosylation states of the protein.

The immunostaining pattern in cryosections of various zones of the male rat kidney is shown in Fig. 3. In the superficial cortex (Fig. 3, A and B), the antibody strongly stained small and large arteries and with varying intensity the BBM of proximal convoluted tubules. The distal tubules and collecting ducts were stained basolaterally and weakly apically. In the outer stripe (Fig. 3C), BBM of the proximal tubule S3 segments was stained more strongly than that in the cortical proximal tubules. In the inner stripe (Fig. 3D), only the small vessels were weakly positive, whereas in the inner medulla (Fig. 3E) smooth muscles in the renal capsule and external blood vessels were brightly stained, whereas collecting ducts and other structures in the papilla remained unstained. Following preincubation of the antibody with the immunizing peptide, the staining of S3 segments in the outer stripe (Fig. 3F), as well as the staining in other zones (not shown), was abolished.

The immunocytochemical data in Fig. 3 indicated axial heterogeneity of rSGLT1-related staining along the proximal tubule; the staining in the outer stripe (S3 segments) was stronger than in the superficial cortex (S1 and S2 segments). This heterogeneity was further tested by immunoblotting of BBM, isolated from the renal cortex and outer stripe of male and female rats (Fig. 4). As shown in Fig. 4A, the immunoblots revealed a stronger 75-kDa protein band in membranes from the outer stripe from both male and female kidneys; by densi-
tometry (Fig. 4B), in both sexes the band in the outer stripe was \(\sim 100\%\) stronger than in the cortex, and it was stronger in females than in males, indicating gender differences. The same pattern, however, was not found for the 40-kDa protein band; the density of this band in this and other experiments was not consistently related to the tissue zone, gender, gonadectomy, or treatment with various hormones (cf. the data in Fig. 10), and its immunoblotting pattern was therefore not described in detail in this and other experiments.

Characterization of the 40-kDa protein band. The different labeling pattern for the 40- and 75-kDa proteins in isolated membranes suggested that, although being blocked by the immunizing peptide, these two proteins may be unrelated. Also, the immunostaining of the basolateral membrane in the cortical distal tubules and collecting duct, as well as the positive staining of smooth muscles in various structures of the jejunum and kidney, where SGLT1 is not known to be localized, indicated the possibility that the observed staining in these localizations may be related to the 40-kDa protein. The next few experiments (Fig. 5 and also see Figs. 6–8) were aimed to resolve this possibility.

BLM were isolated from the male and female kidney cortex, outer stripe, and pooled inner stripe + inner medulla. As shown in Fig. 5A, proteins from the starting homogenate and final membrane preparations were blotted with a polyclonal antibody to the Na-K-ATPase \(\alpha\)-subunit; the relevant \(\sim 100\text{-kDa}\) protein band was strongly enhanced in the final membrane preparations from all tissue zones in both sexes, indicating highly enriched BLM preparations. The two independent BLM preparations from the respective kidney zones of male and female rats were then blotted with the rSGLT1-Ab. As shown in Fig. 5B, the 75-kDa protein band was hardly visible in BLM preparations from the kidney cortex and outer stripe, probably due to the presence of contaminating BBM, and was absent in membranes from the inner stripe + inner medulla. However, a significant 40-kDa protein band was clearly present in BLM preparations from all kidney zones in both sexes, with or without a contaminating 75-kDa band.

From the crude immune serum, we have extracted the antibody against the 40-kDa protein (40 kDa-Ab), and characterized it by Western blotting and immunocytochemistry (Fig. 6). As shown in Fig. 6A, on immunoblots of BBM isolated

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Fig. 3. Immunostaining in cryosections of the male rat kidney with rSGLT1-Ab. In the cortex (A and B), glomeruli (G) were negative, small and large arteries were strongly stained (arrows), BBM of the proximal convoluted tubules (PT) was stained with heterogeneous intensity, whereas distal tubules (DT) and collecting ducts (not shown) were stained largely basolaterally and weakly apically. In the outer stripe (C), BBM of the PT S3 segments was sharply positive, whereas in the inner stripe (D) only small blood vessels (SV) were weakly positive, probably due to reaction with smooth muscles in their walls. In the inner medulla (E), collecting ducts and other structures were negative (*), whereas smooth muscles in the renal capsule (arrowheads) and external blood vessels (arrows) were brightly stained. The immunostaining of S3, as observed in C, was abolished following preincubation of the antibody with the immunizing peptide (F), and the same was found for the staining in other zones (not shown). TALH, thick ascending limb of Henle. Bar = 20 \(\mu\text{m}\).
from the female rat kidney outer stripe the antibody strongly labeled the 40-kDa band and weakly labeled the 75-kDa protein band. Only traces of the labeling remained after preincubation of the 40 kDa-Ab with the immunizing rSGLT1 peptide. By immunocytochemistry, in cryosections of the rat kidney cortex (Fig. 6B) and outer stripe (Fig. 6C), the 40 kDa-Ab stained, albeit weakly, the same structures with the same pattern as the rSGLT1-Ab (cf. Fig. 3): arteries, the apical domain of various proximal tubule segments (outer stripe > cortex), and basolateral and apical domains of distal tubules and collecting ducts. The staining in all these localizations was abolished by preincubating the 40 kDa-Ab with the immunizing rSGLT1 peptide (not shown). Moreover, the staining with the 40 kDa-Ab of the proximal tubule brush border also exhibited zonal (outer stripe > cortex) and gender differences (female > male) (data not shown). In the rat jejunum (Fig. 6D), the 40 kDa-Ab stained the apical domain of the villi and submucosal smooth muscle fibers. Moreover, the same antibody stained distinct structures in the mouse jejunum and kidney cortex; in the mouse jejunum (Fig. 6E), villi were stained weakly apically, whereas smooth muscles in the submucosa and muscularis externa were brightly stained (Fig. 6E). In the mouse kidney cortex (Fig. 6F), arteries were strongly stained and many proximal tubules exhibited a weak apical staining. A stronger intensity but similar pattern of staining in the mouse jejunum and kidney was also observed with the rSGLT1-Ab (data not shown). The staining with both the 40 kDa-Ab and rSGLT1-Ab in these mouse organs was also abolished following preincubation of the antibodies with the immunizing (rSGLT1) peptide (data not shown).

Fig. 4. Zone- and gender-dependent abundances of 40- and 75-kDa protein bands in immunoblots of BBM (60 μg protein/lane) from the kidney cortex and OS (A) and densitometric evaluation of the 75-kDa protein band (B). Shown are the data from 4 independent membrane preparations in male and female rats. In both sexes, the density of the 75-kDa protein band was ~100% stronger in membranes from the OS, proving axial differences. In both tissue zones, the same band was stronger in females, indicating gender differences. The 40-kDa band in this and other experiments (cf. Fig. 10) showed an inconsistent pattern of labeling. \( P < 0.02, \text{a vs. b or c}, P < 0.001, \text{a or c vs. d}, \) Not significant (NS), \( b \) vs. \( c \).

Fig. 5. Abundance of Na-K-ATPase (A) and 40-kDa protein (B) in basolateral membranes (BLM) isolated from the cortex, OS, and pooled inner stripe + inner medulla tissues of male (M) and female (F) kidneys. A: compared with that in the respective tissue homogenates from the same animal (HOM), the Na-K-ATPase α-subunit band (~100 kDa) in isolated BLM from all zones of M and F kidneys was much more prominent, indicating strongly enriched BLM preparations. The bands at ~75 kDa and below represent degradation products of the ~100-kDa protein. Each lane contained 10 μg of protein. B: in 2 independent preparations of BLM from various tissue zones of M and F kidneys (60 μg protein/lane), the 40-kDa protein band was strong and similarly abundant, whereas the abundance of the 75-kDa protein band was negligible. For comparison, both protein bands were abundant in BBM isolated from F kidney outer stripe (FOS BBM).
The positive immunostaining data with the 40 kDa-Ab and rSGLT1-Ab in proximal tubules of the mouse kidney led us to compare immunoblots with BBM isolated from the rat and the mouse kidney. As shown in Fig. 6G, in accordance with the data reported in Fig. 1, in BBM isolated from the rat kidney outer stripe the rSGLT1-Ab strongly labeled both the 40- and 75-kDa protein bands, whereas in BBM from the mouse kidney the same antibody strongly labeled the 40-kDa and only weakly the 75-kDa protein band (−P). All the bands were abolished or strongly diminished following preincubation of the rSGLT1-Ab with the immunizing peptide (+P). As further shown in Fig. 6G (ACTIN), in BBM from both rat and mouse kidney the 40-kDa protein band colocalized with the α-actin band (42 kDa), suggesting that the 40 kDa-Ab-related immunoactivity may cross-react with α-actin, known to be present in microvilli and smooth muscles. This possibility was tested by double-staining with the anti-α-actin antibody and 40 kDa-Ab in cryosections of the kidney cortex (Fig. 7, A1–A3) and luminal domains of the jejunum in female rats (Fig. 7, B1–B3). Accordingly, in the kidney cortex (Fig. 7A1) actin was brightly stained in the glomerulus (G), artery (arrowhead), and proximal tubule brush border (arrows), whereas the 40 kDa-Ab stained the artery and proximal tubule brush border but not the actin-rich glomerulus (Fig. 7A2), displaying the yellow colocalization color only in the artery and proximal tubule brush border (Fig. 7A3). In the apical domain of the rat jejunum, the actin antibody brightly stained the brush border (long arrow) and lateral membrane of the reabsorptive cells (short arrows), whereas the 40 kDa-Ab clearly stained only the brush border, exhibiting a yellow colocalization color only in the brush border (Fig. 7, B1–B3, respectively). The same picture was obtained with the rSGLT1-Ab (data not shown). This experiment thus indicates that the 40 kDa-Ab labels/stains a protein differently from α-actin or its isoforms.

Following the information obtained in the preceding experiments, that both the rSGLT1-Ab and 40 kDa-Ab stained smooth muscles in the rat and mouse kidney and small intestine, we performed immunostaining studies using cryosections of the rat aorta and uterus, and immunoblotting studies of total cell membranes isolated from the same tissues using the rSGLT1-Ab. These organs are rich in smooth muscles, but SGLT1 protein was not expected to be present there. As shown in Fig. 8, A and C, the antibody strongly stained smooth muscles in the rat aorta, uterus, and the uterine artery, and this staining was strongly diminished or abolished following preincubation of the antibody with the immunizing (rSGLT1) peptide (Fig. 8, B and D). In immunoblots, however, in accordance with the data in Figs. 1 and 6, the rSGLT1-Ab labeled both 40- and 75-kDa protein bands in rat kidney BBM, whereas in total cell membranes from the rat aorta and uterus,
even after a prolonged staining only the 40-kDa band was labeled (Fig. 8E; −Peptide). The bands were abolished with the peptide-blocked antibody (Fig. 8E; +Peptide). This experiment thus indicates that the 40-kDa protein, which is present in smooth muscles of various organs, as well as in isolated renal BBM and BLM and intestinal BBM, is unrelated to the 75-kDa protein.

Gender differences in the expression of rSGLT1 along the rat nephron. The Western blotting data in Fig. 4 indicated gender differences in the abundance of the 75-kDa protein in BBM from the kidney cortex and outer stripe. These immunoblotting data were confirmed by immunocytochemistry performed in cryosections of male and female tissues with the blotting data were confirmed by immunocytochemistry performed in cryosections of male and female tissues with the peptide-blocked antibody (Fig. 8F). The same data were obtained by using rSGLT1-Ab instead of 40 kDa-Ab (not shown).

Effect of gonadectomy and replacement therapy with sex steroids. To determine the sex hormone(s) responsible for the observed gender differences in rSGLT1-Ab-related immunoreactivity in rat kidneys, we performed Western blotting in BBM or total cell membranes from the kidney cortex and outer stripe, and immunostaining in cryosections of the same kidney zones in prepubertal (25 day old) rats and in gonadectomized adult rats without and with therapy with various sex hormones.

In prepubertal rats, the overall immunostaining of the tubules in the kidney cortex and outer stripe resembled that in adult male rats, with a similar pattern and intensity in both sexes (data not shown). Furthermore, the density of the 40- and 75-kDa protein bands in total cell membranes from the cortex and outer stripe in prepubertal male and female rats was similar to that in total cell membranes from the same tissue zones in adult male rats, exhibiting no gender differences (blots not shown). These experiments thus indicated that gender differences in rSGLT1-related immunoreactivity in the rat kidney occur after puberty.

To study effects of gonadectomy and hormonal treatment, the adult animals were either sham-operated or gonadectomized, left to recover for 8 days, and then treated with either oil or various gonadal hormones for another 8 days. Compared with the data in sham-operated females, ovariecetomy caused no significant change in the density of both 40- and 75-kDa protein bands in immunoblots of BBM isolated from the kidney cortex or outer stripe, and no visible change in either the staining pattern or intensity in tissue cryosections (data not shown). These experiments thus indicated that the ovarian hormones may not be responsible for the observed gender differences. On the contrary, castration in males caused a ~5- and 3-fold increase in the density of the 75-kDa protein band in total cell membranes from the kidney cortex and outer stripe, respectively (Fig. 10, A and B). This elevated abundance of the 75-kDa protein in membranes from both zones of castrated, oil-treated rats was not affected by the treatment with estradiol or progesterone. In testosterone-treated castrated animals, however, abundance of the 75-kDa protein was significantly
of Na\(^+\) gradient-driven, phlorizin-inhibitable uptake of \(^{3}H\)galactose in BBM vesicles isolated from the kidney cortex and outer stripe of the male and female kidney. The data (Fig. 12) revealed that 1) uptake of \(^{3}H\)galactose in the vesicles from the outer stripe of male and female kidneys was, respectively, 80 and 110% higher than in the vesicles from the cortex, thus proving the presence of axial differences; and 2) uptake in the respective vesicle preparations from the female kidneys was 50 (cortex) and 70% (outer stripe) higher than in the vesicles from the male kidneys, thus proving the presence of gender differences in the transport of \(^{3}H\)galactose. These uptake data showing relative expression of the specific transport in the vesicles from various kidney zones are, therefore, a very good match with the immunoblotting data in Fig. 4, which show a similar relative abundance of the 75-kDa protein in BBM from the same zones.

**DISCUSSION**

Previous transport studies in isolated proximal tubules and BBM vesicles from rabbit kidney (3, 28–30), as well as Northern blot and in situ hybridization studies in rabbit and rat kidney tissues (15, 21, 35), indicated that the proximal tubule S3 segments in the outer stripe and medullary rays are the predominant site of SGLT1. However, this localization of SGLT1 along the mammalian nephron so far has not been consistently confirmed by immunochimical experiments. In rare studies with polyclonal antibodies, a putative SGLT1 protein of 77 kDa was immunolocalized to the brush-border membrane along the entire proximal tubule in rats (26), whereas the results of Pajor et al. (20) indicated that the protein was more abundant in the outer stripe of the rabbit kidney.

In this report, we used four different methods (immunoblotting, immunocytochemistry, uptake studies, and Northern blotting) to study the expression of rSGLT1 in the distinct kidney zones of male and female rats. The data indicate that SGLT1 in the rat kidney is represented by a (possibly glycosylated) 75-kDa protein, which is expressed predominantly in the proximal tubule S3 segments, where it exhibits gender differences (female > male) caused by the inhibitory action of androgen hormones.

Our immunochimical studies were performed with a recently generated rSGLT1 peptide-specific polyclonal antibody (8, 9). In Western blots of isolated brush-border or total cell membrane from various kidney zones, in our hands the antibody labeled two major bands with relative mobilities of 40 and 75 kDa. The compact 40-kDa band was not zone, gender,
gonadectomy, or hormone treatment dependent; rather, it varied in density in day-to-day membrane preparations. The origin of the 40-kDa protein band is unclear. Our double-labeling/staining experiments indicated that this protein is not \( \alpha \)-actin; it showed similar mobility to, but a different staining pattern from, actin. There are several possibilities to explain the nature of the 40-kDa protein band.

1) The 40-kDa protein may possibly represent a truncated proteolytic product of SGLT1 because the rSGLT1-Ab and 40 kDa-Ab exhibited a largely similar pattern of labeling on immunoblots and immunostaining in cryosections, which was prevented by the same immunizing peptide. However, the presence of a significant 40-kDa band in BLM preparations from various kidney zones and in total cell membranes from the rat aorta and uterus, where the 75-kDa protein was completely negative, makes this possibility very unlikely. In addition, it is unlikely that truncated or complete SGLT1 transporters are located in both basolateral and apical cell membrane of distal tubules and collecting ducts; this would point to the presence of a \( \text{Na}^+ \)-dependent mechanism of accumulation of monosaccharides into the respective cells from both cell sides, a so far unrecognized phenomenon in renal physiology.

2) The 40-kDa protein may be a splice variant of rSGLT1, the expression of which is neither gender nor sex hormone dependent, but we have not performed the sequence analysis of both proteins to (dis)prove it. 3) The 40-kDa protein may be completely unrelated to rSGLT1, but it may be recognized by the rSGLT1-Ab due to the presence of similar antigenic domains in both the 40- and 75-kDa proteins. We favor the last possibility, considering the fact that the immunostaining with both the rSGLT1-Ab and 40 kDa-Ab exhibited different staining intensities (rSGLT1-Ab > 40 kDa-Ab) but a similar pattern of zonal (outer stripe > cortex) and gender (female > male) differences in the rat kidney. Both proteins may either colocalize to the same or reside in different structures in the rat kidney, including the BBM of the proximal tubules and basolateral and apical membranes of the distal tubules and collecting ducts, and smooth muscle cells in the renal capsula and blood vessels, as well as in the BBM and smooth muscles in the rat and mouse small intestine. The phenomenon by which the 40 kDa-Ab, extracted from crude immune serum, on the blot of BBM strongly labels the 40-kDa and weakly labels the 75-kDa protein band, and by immunocytochemistry exhibits the same, rSGLT1-peptide-blockable staining pattern as with the rSGLT1-Ab, makes it hard to
distinguish whether both proteins are localized in the same or different renal structures. However, the ubiquitous localization of the 40-kDa protein in the rat and mouse kidney and intestine, as well as in the rat aorta and uterus, suggests that this protein belongs to a group of gender-independent, structural proteins that at least partially share the amino acid sequence with the immunizing rSGLT1 peptide. In this regard, by using the database search program BLAST we have compared the amino acid sequence of the rSGLT1 peptide with sequences in other relevant proteins and found two recently described proteins in rat, mouse, and human tissues, under different names but with highly homologous (>87%) sequences, as plausible

Fig. 10. Immunoblots with rSGLT1-Ab of the TCM (80 μg protein/lane) from the kidney cortex and OS of male rats with effect of castration and treatment with various sex hormones (A) and densitometric evaluation of the 75-kDa protein band (B). Shown are blots from 2 independent experiments with 2 animals in each experimental group. In the cortical membranes, compared with the band in sham-operated, oil-treated animals (SHAM+O), the density of the 75-kDa protein band was strongly and similarly enhanced in castrated rats treated with oil (O), testosterone (T), estradiol (E), or progesterone (P). In the OS membranes, the strongly enhanced 75-kDa protein band in castrated, oil-treated rats was inhibited by 50% following the treatment of castrated animals with testosterone, but not with estradiol or progesterone. The 40-kDa protein band in the membranes from both kidney zones showed no particular castration- and hormone treatment-dependent pattern. B: bars represent the densitometric data of the 75-kDa protein band pooled from the experiments shown in A (n = 4). *P < 0.01, a vs. b, P < 0.05, a vs. c. *P < 0.05, b vs. c. NS, b vs. b.

Fig. 11. Immunostaining with rSGLT1-Ab in cryosections of the kidney cortex and OS of control (sham-operated + oil-treated) and variously treated castrated rats. In the cortex, the pattern of staining in various tubules was generally weak and similar in all experimental groups, except that the intensity of BBM staining in the S3 segments in medullary rays followed that in the S3 segments of the OS (not shown). In the OS, the intensity of BBM staining in the S3 segments was strongly enhanced in castrated, oil-treated rats, whereas the treatment of castrated animals with testosterone strongly downregulated the staining intensity. In castrated rats treated with estradiol or progesterone, the staining intensity in the S3 BBM remained high. Bar = 20 μm.
related immunostaining (outer stripe S3 segments in the outer stripe and medullary rays). This predominantly detected in brush border of the proximal tubule cortex, whereas in tissue cryosections, the immunostaining was protein was more abundant in the outer stripe than in the cell membranes, in both male and female kidneys, the 75-kDa rate of SGLT1-mediated glucose/galactose transport (3, 28–40). The complexity of the 75-kDa band was strong in BBM from the rat jejunum and uterus, e.g., in tissues in which rSGLT1 was not expected to, band was absent in total cell membranes from the rat aorta and 40-kDa protein band on the blot and contributing to the overall immunostaining of various structures in rat and mouse kidney existence in different glycosylation states. 

The cloned rSGLT1 protein has 664 amino acid residues and completely correlated with the staining intensity of the gender, gonadectomy, and sex hormone-treatment dependent and completely correlated with the staining intensity of the brush border in S3 segments. The following line of evidence indicates that the 75-kDa protein band is related to rSGLT1. 1) The cloned rSGLT1 protein has 664 amino acid residues and one N-glycosylation site (Refs. 15 and 34 and references therein); by Western blotting, these characteristics should yield a glycosylated protein band of at least 73 kDa. In previous publications, rSGLT1 was identified as a broad 70- to 80 (8, 9)- or 77-kDa protein band (26). The complexity of the 75-kDa band in our isolated renal BBM indicates that the protein may exist in different glycosylation states. 2) The 75-kDa protein band was absent in total cell membranes from the rat aorta and uterus, e.g., in tissues in which rSGLT1 was not expected to be, whereas the band was strong in BBM from the rat jejunum and renal outer stripe, e.g., in cell membranes that exhibit a high rate of SGLT1-mediated glucose/galactose transport (3, 28–30, and the current study). 3) By immunoblotting of isolated cell membranes, in both male and female kidneys, the 75-kDa protein was more abundant in the outer stripe than in the cortex, whereas in tissue cryosections, the immunostaining was predominantly detected in brush border of the proximal tubule S3 segments in the outer stripe and medullary rays. This zonal-dependent abundance of the rSGLT1 protein and the related immunostaining (outer stripe > cortex) were a complete match to the phlorizin-sensitive uptake of d-[3H]galactose in isolated BBM vesicles, which in both sexes was stronger in membranes from the outer stripe, and with the predominant localization of the rSGLT1-specific mRNA in proximal tubule S3 segments in the outer stripe and medullary rays, which agrees with previous in situ hybridization studies (15). However, possibly due to some experimental error, our data did not confirm the zonal-dependent expression of rSGLT1-related mRNA in male kidneys, but such an expression, threefold higher in the outer stripe than in the cortex, was clearly demonstrated in female kidneys. 4) Clear gender differences (females > males) were observed for density of the 75-kDa protein band in BBM and total cell membranes from both kidney cortex and outer stripe, intensity of immunostaining in S3 segments in the outer stripe and medullary rays, uptake of d-[3H]galactose in BBM vesicles from the cortex and outer stripe, and expression of rSGLT1-specific mRNA (albeit only in the outer stripe). Because immunostaining data proved gender differences only in the brush border of S3 segments in the outer stripe and medullary rays, gender differences in the density of the 75-kDa protein band and in the uptake of d-[3H]galactose in the cortical membranes can be attributed to the BBM that originate from S3 segments residing in medullary rays. These tubules are, therefore, homogenized with the bulk of the cortical tissue that contains S1 and S2 segments with little or no rSGLT1. Furthermore, 5) the experiments with gonadectomy in male and female rats, as well with hormonal treatment of castrated males, clearly show that the density

![Fig. 12. Phlorizin-sensitive D-[3H]galactose uptake in BBM vesicles from the kidney cortex (C) and OS of male and female rats. Shown are the data obtained with the number of independent vesicle preparations indicated in brackets. P < 0.05, a vs. b, NS, b vs. c. P < 0.01, a vs. d and c vs. d. P < 0.02, b vs. d.](Image 1)

![Fig. 13. Northern blot of mRNA, isolated from the kidney cortex and OS of male and female rats and labeled with specific, radiolabeled probes for rSGLT1 and GAPDH (A) and ratio of the band densities (rSGLT1/GAPDH). B: data for 3 independent mRNA preparations from tissue samples that had been pooled from 2 (cortex) or 3 (OS) rats. NS, a vs. b or c and b vs. c. P < 0.02, a vs. d. P < 0.001, b and c vs. d.](Image 2)
of the 75-kDa protein band in isolated membranes and the relevant immunostaining completely match in the outer stripe, whereas in the cortex, these two parameters are related to the expression of the protein in S3 segments that reside in medullary rays. Moreover, both rSGLT1-related parameters in membranes and tissue samples from the outer stripe were weak and exhibited no gender differences in prepubertal male and female rats and were not affected by ovariectomy or by treating castrated rats with estradiol or progesterone, thus indicating that zonal and gender differences in the expression of rSGLT1 appear after puberty and ovarian hormones play no significant role in the expression of rSGLT1 in S3 segments of the rat kidney. On the contrary, both parameters were upregulated in castrated males, and their elevated expression was downregulated by treating castrated animals with testosterone, thus pointing to the androgen hormones as inhibitors of rSGLT1 expression in S3 segments of the adult male kidney. Therefore, whereas zonal differences in the expression of rSGLT1 (outer stripe > cortex) come from the predominant localization of the functional transporter and its mRNA in the proximal tubule S3 segments, gender differences (females > males) are caused by the inhibitory effects of androgen hormones on the transporter and its mRNA expression in the same tubule segments in male rats.

With its gender-dependent expression, rSGLT1 behaves similarly to the recently described organic anion transporters OAT1 and OAT3 in the rat kidney (Ref. 17 and references therein), organic anion transporter protein OATP1 in the rat and mouse kidney (12, 18), and organic cation transporter OCT2 in the rat kidney (31, 32). However, whereas the expression of these transporters is strongly upregulated by androgens and weakly downregulated by estrogens at the level of mRNA, protein, and function, the expression of rSGLT1 in the rat S3 is downregulated by androgens and unaffected by estrogens. Examples of a similar, androgen-dependent downregulation of the protein activity/expression can be found for cytosolic carbonic anhydrase in the rat kidney (25) and mitochondrial ornithine aminotransferase in the mouse kidney (16), which are increased by castration and decreased by testosterone treatment; however, similar data for the cell membrane-bound protein/transporter could not be found in the current literature.

Previously, we demonstrated by the rapid filtration technique strong gender differences (M > F) in Na\(^+\)/H\(^+\) exchanger and Na\(^+\)-glucose cotransport in brush-border membrane vesicles isolated from the mouse kidney cortex; the rates of both transports were higher in membrane vesicles from the male kidney, and testosterone treatment of castrated males or intact females increased these rates two- to threefold (19). The same effects were not observed in brush-border membrane vesicles isolated from the kidney cortex of male and female rats. On the one hand, these data indicate that the SGLT transporters in the mouse and rat cortical proximal tubules may be differentially regulated by sex hormones (species differences?) and on the other hand that in the mouse kidney the cortical SGLT (presumably mSGLT2 in S1 and S2 segments) may be regulated differently from the SGLT in the outer stripe (mSGLT1 in S3 segments). However, in this report we did not study gender differences and zonal expression of SGLT1 in the mouse kidney, and possible differences in the expression of related proteins and mRNAs in the mouse kidneys cannot be concluded.

Our data for castration and sex hormone treatments in castrated rats showed that the expression of rSGLT1 in proximal tubule S3 segments is exclusively affected by androgens, whereas estrogens and progesterone had no effect. Whether this downregulation of renal rSGLT1 expression by androgens resulted from transcriptional, translational, or both mechanisms is unknown at the present. We also do not know whether this effect is mediated by androgen receptors (AR) in S3 segments; AR have been localized to the nuclei in proximal and distal tubule cells of the rat kidney (27). By checking the rSGLT1 genomic sequence (accession no. AC112342), we found out that rSGLT1 has two androgen receptor elements (ARE), one at the position -6481 to -6495 bp and the second one at the position -5779 to -5765 bp. In addition, this sequence has two estrogen-related receptor elements (ERRE). The presence of ARE in the genomic sequence indicates that the rSGLT1 gene possesses a structural binding component for possible interaction with androgens. However, the human SGLT1 genomic sequence (accession no. Z74021) has only one ERRE, but no ARE at all, suggesting that similar gender differences in renal SGLT1 expression, driven by androgen inhibition, are unlikely in humans. Accordingly, in a 1930 publication by Harding and Moberley (10) the urinary excretion of galactose was studied in men and women after the ingestion of galactose, but the final data were inconclusive; although on the average, women excreted more galactose than men on the same intake, the individual variations were too large for any clear-cut conclusion regarding gender differences. Similar studies in rats have not been reported to our knowledge, and possible implications of gender differences in the renal expression of rSGLT1 on the reabsorptive capacity for glucose and galactose in the rat kidney are unclear. However, in male and female mice infused with progressively increasing amounts of glucose, the renal threshold for glucose excretion was three- to fourfold above the level of normal glucose concentration in the blood plasma of both sexes, indicating that possible gender differences in the SGLT1 expression in proximal tubules did not significantly contribute to the overall capacity for glucose/galactose reabsorption.

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