Fingerprints of hSGLT5 sugar and cation selectivity

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Ghezzi C, Gorraitz E, Hirayama BA, Loo DD, Grempler R, Mayoux E, Wright EM. Fingerprints of hSGLT5 sugar and cation selectivity. Am J Physiol Cell Physiol 306: C864–C870, 2014. First published February 26, 2014; doi:10.1152/ajpcell.00027.2014.—Sodium glucose cotransporters (SGLTs) mediate the translocation of carbohydrates across the brush border membrane of different organs such as intestine, kidney, and brain. The human SGLT5 (hSGLT5), in particular, is localized in the kidney where it is responsible for mannose and fructose reabsorption from the glomerular filtrate as confirmed by more recent studies on hSGLT5 knockout mice. Here we characterize the functional properties of hSGLT5 expressed in a stable T-Rex-HEK-293 cell line using biochemical and electrophysiological assays. We confirmed that hSGLT5 is a sodium/mannose transporter that is blocked by phlorizin. Li+ and H+ ions were also able to drive mannose transport, and transport was electrogenic. Our results moreover indicate that substrates require a pyranose ring with an axial hydroxyl group (–OH) on carbon 2 (C-2). Compared with Na+/glucose cotransport, the level of function of Na+/mannose cotransport in rat kidney slices was low.

SGLT5; mannose; kidney

COTRANSPORTERS ARE MEMBRANE transport proteins that use the electrochemical potential gradients (Na+ and H+) to transport sugars, amino acids, neurotransmitters, and osmolytes into the cell against their concentration gradient (23). The Na+–dependent glucose cotransporters (or SLC5) family include 12 human members, 6 of which have the capacity to bind and/or transport monosaccharides (see Ref. 28 for an extensive review). SGLT1 is mainly expressed in the small intestine where it transports glucose and galactose across the brush border membrane (28), SGLT2 is found in the proximal tubule of the kidney (29) and brain (30), SGLT3 is a sodium-dependent mannose and glucose transporter expressed in the small intestine, kidney, and other organs (26); and SGLT6, expressed in the brain, kidney, and intestine, transports D-chiro-inositol and also recognizes D-glucose (17).

For long time little was known about hSGLT5, and only recently a initial functional characterization has been performed (9). Real-time PCR experiments, performed on different tissue samples, showed that hSGLT5 is mainly expressed in the kidney, resembling the hSGLT2 expression pattern (28). The protein was stably transfected in HEK T-Rex cells, and radiotracers uptake experiments showed that it transports mannose, fructose, glucose, and galactose in a Na+–dependent manner. The highest uptake was measured for mannose followed by fructose whereas glucose and galactose showed very low specific uptake rates (9).

A new class of antidiabetes drugs that inhibit specifically hSGLT2 has been developed with the aim of reducing glucose plasma concentration by increasing the amount that is excreted in the urine (3). As it has been shown that in diabetic patients mannose plasma levels are high and correlate with increased glucose levels (20, 24, 25), it is particularly important to understand the role of mannose metabolism. Since hSGLT5 shows the same tissue distribution as hSGLT2, Grempler et al. (9) tested different SGLT2 inhibitors (e.g., Canagliflozin and Dapagliflozin). The results showed that, as the other members of the family, hSGLT5 is phlorizin sensitive and that monosaccharide uptake via SGLT5 is only weakly inhibited by all the hSGLT2 inhibitors tested.

The fact that the study by Grempler at al. (9) indicated that SGLT5 is one of the specific transporters that contribute to the control of plasma mannose levels by regulating its handling in the kidney suggests that SGLT5 mechanism of action should be characterized in more detail. In the present study we describe in detail the kinetics of mannose transport, substrate and cation specificity, and electrogeneity.

MATERIALS AND METHODS

Reagents and solutions. For radiotracer uptake experiments, the extracellular solution [0.9% phosphate-buffered, glucose-free saline (PBS)] contained the following (in mM): 137 NaCl, 10 Na2HPO4, 1.76 NaH2PO4, and 2 KCl; pH 7.4 adjusted with Tris. For whole cell patch-clamp experiments, the standard extracellular solution (Na+ buffer) contained the following (in mM): 150 NaCl, 1 CaCl2, 1 MgCl2, and 10 HEPES pH 7.4 adjusted with Tris. For Na+ free solution, NaCl was equimolarly replaced with choline Cl or LiCl and pH was adjusted to 7.4 with Tris or to 5 with MES. The standard intracellular solution (pipette solution) contained the following (in mM): 145 CsCl, 5 NaCl, 11 EGTA, and 10 HEPES pH 7.4.

Cell culture and transfection. T-Rex-293 cells stably transfected with hSGLT5 were provided by Boehringer Ingelheim (9). Cells were cultured in DMEM (CELLGRO, Manassa, VA) containing 10% FBS (Valley Biomedical Products, Winchester, VA), 600 μg/ml geneticin, and 5 μg/ml blasticidin (Invitrogen). Cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO2-95% air, passaged (1:10) every 5 days, and seeded in 6-well plates. Twenty-four hours before the experiments, hSGLT5 transcription was induced by addition of 1 μM/ml tetracycline (Sigma-Aldrich, St. Louis, MO).

Radiotracer uptake. Sugar uptake was measured in HEK 293T cells expressing hSGLT5 as described previously (15). In brief, cells were plated on poly-lysine-treated 24-well plates and activated with 1 μM/ml tetracycline 24 h before the assay. Cells were incubated for 30 min at 37°C in 50 μM [3H]-[α-L-methyl-14C]glucopyranoside (Perkin Elmer, Walthman, MA) or [14C]mannose (Moravek Biochemicals, Brea, CA), washed in cold choline buffer three times, and solubilized in 1% Triton-X-100 (Sigma-Aldrich). A sample was assayed using scintillation counting. To minimize the potential contamination due to...
GLUT transporters, transport assays were carried out in presence of 10 μM cytochalasin B (Sigma-Aldrich).

For each tested condition, the sample size was n = 3–4 wells and each experiment was repeated at least twice. Uptakes were measured in the presence and absence of 100 μM phlorizin and the SGLT-specific uptake was taken as the phlorizin sensitive component. Uptakes were expressed as picomoles per minute per microgram total protein and means ± SE.

For insulin activation experiments, insulin (Sigma) was added from a 100 μM stock to give the final concentration of 100 and 400 pM. Insulin was added to complete DMEM medium, and cells were incubated for 2 h at 37°C and controlled O2/CO2 before the transport assay (8).

For kinetic analysis, the Na+ concentration ([Na+]) was varied from 0 to 150 mM and data were plotted as a function of [Na+] and fitted with the modified Hill equation of the form

\[ v = \frac{v_{max}([S]_o^n)}{([S]_o^n) + (K_{Na}^{0.5})^n} \]  

where \( v_{max} \) is the maximal velocity of transport, \([S]_o\) is the external substrate concentration, \( K_{Na}^{0.5} \) is the substrate concentration at half maximal current, and \( n \) is the Hill coefficient.

Sugar specificity of hSGLT5 was determined by measuring the effect of competing sugars [0–100 mM D-mannose, D-xylose, 2-deoxy-D-glucose (2-DG), D-ribose, and D-lyxose] on 50 μM [14C]mannose uptake.

Whole cell patch-clamp recording. Whole cell patch-clamp experiments on T-Rex 293-hSGLT5 cells were carried out as described previously (15). In brief, experiments were performed 24 h after transfection. hSGLT5 plasmids were linearized with SalI and BstE II to remove the hSGLT1 coding sequence and 15-bp extensions that were complementary to the ends of the linearized pBluescript vector. hSGLT5 was amplified with a high-fidelity polymerase and 3′-untranslated regions from hSGLT1. Briefly, the pBluescript plasmid was digested with SalI and BstE II to remove the hSGLT1 coding sequence and then purified. PCR primers were designed for hSGLT5 containing 15-bp extensions that were complementary to the ends of the linearized pBluescript vector. hSGLT5 was amplified with a high-fidelity polymerase, gel purified, and then cloned into pBluescript using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA). Competent cells were transformed and colonies were selected in a medium with ampicillin. Plasmid DNA was prepared using purification kits by Qiagen. The fidelity of the new clone was verified by restriction analysis and sequencing. hSGLT5 plasmids were linearized with XbaI, and cRNA was produced in vitro using the mMessage-Machine kit T3 (Ambion).

The expression of hSGLT5 in oocytes follows the protocols developed for SGLT1 (11, 12, 16, 22, 27). Briefly, hSGLT5 cRNA is injected into oocytes, the cells are cultured for 3–5 days at 18°C. Control studies were carried out with hSGLT1 cRNA.

Radioactive tracer uptakes in rat kidney slices. Sprague-Dawley rats were used in all experiments in accordance with institutional guidelines and with the approval of the Institutional Animal Care and Use Committee. Rats were euthanized by intravenous injection of pentobarbital sodium (Sigma-Aldrich). Kidneys were removed, decapsulated and placed into ice-cold sodium buffer. Coronal slices (300 μM) were made using a Vibratome (series 1000, Pelco 101) (2, 30). Slices were incubated for 30 min at 22°C in sodium buffer or choline buffer containing 10 μM cytochalasin B and 50 μM [14C]mannose or D-[α-methyl-14C]glucopyranoside ([14C]Glu-MDG) in the absence or presence of 100 μM phlorizin. After incubation, slices were washed in ice-cold sodium buffer, weighted, dissolved in NCS tissue solubilizing fluid (MP Biomedical, St. Ana, CA), and assayed using a liquid scintillation counter. Uptakes are expressed as picomoles per milligrams of tissue.

Statistical analysis. All data were tested for significance using ANOVA and unpaired Student’s t-test where appropriate. Only values with \( P < 0.05 \) were considered as significant.

RESULTS

hSGLT5 transports mannose and is phlorizin sensitive. Grempler et al. (9) previously showed that hSGLT5 is a sodium-dependent monosaccharide transporter with a higher affinity for mannose and fructose over glucose and galactose. As a starting point, we confirmed that hSGLT5 transported mannose at higher rate than α-MDG. Figure 1A shows the results of a typical experiment: the initial rate of [14C]mannose uptake was seven times greater than [14C]α-MDG. 100 μM phlorizin reduced mannose and α-MDG uptake to the same background level. These experiments were carried out in the presence of 10 μM cytochalasin B to block phlorizin-insensitive mannose transport by endogenously expressed GLUTs in HEK-293T cells (1). We previously reported that glucose transport by hSGLT2, but not hSGLT1, was dramatically increased by phlorizin (8). As observed for hSGLT1 we could not detect any effect of 100 or 400 pM insulin on mannose transport mediated by hSGLT5 (Fig. 1B). As was the case for hSGLT2, we were also unable to detect any phlorizin-sensitive [14C]mannose or α-MDG uptake into Xenopus laevis oocytes injected with hSGLT5 cRNA (data not shown).

Cation and sugar specificity of hSGLT5. Mannose uptake with 150 mM sodium in the extracellular solution was phlorizin sensitive and substantially higher than the uptakes when NaCl was replaced with either LiCl or choline Cl (Fig. 2A). When the pH was reduced to 5.0 in choline Cl, the phlorizin-sensitive mannose uptake increased to ~30% of that in NaCl at pH 7.4 (Fig. 2A). Phlorizin (100 μM) inhibited mannose uptake in Na, Li, and choline to the same level. These results indicate that H+ and Li+ can drive mannose transport by hSGLT5.

[14C]mannose uptake was measured as a function of extracellular Na+ concentration, replacing NaCl with choline Cl (Fig. 2B). Uptake increased with external [Na+] and approached saturation at 150 mM NaCl with a Hill coefficient of 1 and an apparent affinity \( (K_{0.5}) \) of 35 mM.

The sugar selectivity of hSGLT5 was tested by measuring the ability of 100 mM sugars to inhibit 50 μM d-[14C]mannose uptakes (Fig. 3, A and B). Mannose (100 mM) inhibited radiotracer uptake 100%, as expected, since the \( K_{0.5} \) for mannose transport by hSGLT5 is 0.45 mM (9). D-xylose (100 mM) inhibited 50%, 100 mM d-ribose 34%, and 100 mM D-lyxose 20%. This indicates that the inhibition constants \( (K_i) \) for xylose, ribose, and xylene are 100 mM or higher.

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Previous experiments on hSGLT1 and hSGLT2 have shown that the essential requirements for substrate interaction and transport are a pyranose ring with an equatorial hydroxyl group on carbon 2 (C-2) (see for a review Ref. 28). Removal of this hydroxyl group reduced the apparent affinity of 2-DOG for hSGLT1 by \( \frac{1}{100} \) or in nontransfected HEK-293T cells (data not shown). The mannose currents were sensitive to voltage (Fig. 4B). The hSGLT5 mannose current in the same cells as Fig. 4A was curvilinear, i.e., increased in a nonlinear fashion between \(+50\) and \(–25\) mV and then linearly between \(–25\) and \(–150\) mV.

When expressed in *Xenopus laevis* oocytes or HEK-293T cells, hSGLT1 shows presteady-state currents in response to step changes in membrane potential, i.e., hSGLT1 capacitive transients, proportional to the number of transporters in the plasma membrane (18, 29). Similar to hSGLT2 (15), no hSGLT5 presteady-state currents were observed in either the presence or absence of mannose when the membrane voltage was jumped within 2 ms between the holding potential, \(–60\) mV, and \(–150\) or \(+50\) mV. The absence of presteady-state currents is either due to 1) faster transients that can be recorded by our whole cell patch clamp, or 2) low expression of hSGLT5 in the plasma membrane.

Radioactive tracer uptakes in rat kidney slices. The hSGLT5 gene is exclusively expressed in the kidney cortex, but the localization of the protein and its physiological role remain unknown. We, therefore, performed mannose uptake experi-

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**Fig. 1.** Radioactive tracer flux experiments in the human Na\(^{+}/\)\(d\)-glucose cotransporters 5 (hSGLT5). A: \(d\)\-[\(\alpha\)-methyl\(\L^1\)]glucopyranoside (\(\alpha\)-MDG; 50 \(\mu\)M) and \(L\)\-[\(\alpha\)\-[\(\alpha\)]mannoside (Mann; 50 \(\mu\)M) uptake were measured in hSGLT5 stably transfected human embryonic kidney 293 (T-Rex 293) cells at 37°C. Uptake is expressed as quantity of tracer (pmol) per min per \(\mu\)g protein. Uptake measured in control conditions (150 mM Na\(^{+}\), white bars) was significantly \((P < 0.05)\) bigger than in the presence of the inhibitor phlorizin (Pz; 100 \(\mu\)M, grey bars). Bars are means \(\pm\) SE; \(n = 4\) wells. B: \[\(\L^1\)\]mannose uptake was measured in control conditions (white bar) and after incubation with 100 \(\mu\)M (grey bar) or 400 \(\mu\)M (black bar) insulin. Bars are means \(\pm\) SE; \(n = 4\) wells.

**Fig. 2.** Cation dependence of mannose uptake. A: \[\(\L^1\)\]mannose (50 \(\mu\)M) uptake was measured in presence of 150 mM Na\(^{+}\) in the extracellular solution or replacing NaCl with choline Cl. (pH 7.4 and 5) or LiCl. For each condition we measured uptake in the absence (white bars) or presence (grey bars) of 100 \(\mu\)M phlorizin. Uptake is expressed as quantity of tracer (pmol) per min per \(\mu\)g protein. Bars are means \(\pm\) SE; \(n = 4\) wells. B: \(\L^1\)mannose (50 \(\mu\)M) uptake was measured as a function of Na\(^{+}\) concentration in the extracellular solution. Data points were fitted with Eq. 1 to get the substrate concentration at half maximal current (\(K_{0.5}\)). Data are means \(\pm\) SE; \(n = 4\) wells.
ments in rat kidney slices in the absence and presence of phlorizin (Pz), to determine if SGLT5 is functionally active in the plasma membrane of this tissue. First, we carried out control experiments measuring the uptake of \([^{14}C]H_9251\)-MDG. As expected, the uptake was sodium dependent, and significantly reduced in the presence of 100 \(M\) phlorizin (Fig. 5A). These results confirmed the viability of the slices by showing functional expression of a Na-dependent phlorizin-sensitive \([^{14}C]H_9251\)-MDG transporter, SGLT1 and/or SGLT2, in rat kidney plasma membranes. In contrast, 50 \(M\) mannose uptake was not inhibited by 100 \(M\) of phlorizin (Fig. 5B), suggesting that the functional expression of SGLT5 is low relative to that for SGLT1/SGLT2. Mannose uptake was significantly reduced in the absence of Na\(^{+}\) (\(P \leq 0.05\)) as was noted for mannose uptake into renal brush border membranes from SGLT5\(^{-/-}\) mice (7).

**DISCUSSION**

In recent years, specific hSGLT2 inhibitors have been developed with the aim of lowering plasma glucose levels of type II diabetic patients by decreasing the amount of glucose reabsorbed in the kidney (3). Although hSGLT2 is considered the main transporter responsible for glucose handling in the kidney, different transporters in the SLC5 (or SGLT) family may also be involved in the regulation of renal sugar reabsorption. For example, hSGLT1, expressed in the late proximal tubule, is involved in salvaging any remaining glucose from the glomerular filtrate that is not absorbed by hSGLT2 in the early proximal tubule (21).

These drugs have been designed to target hSGLT2 over hSGLT1, but their interaction with other members of the SLC5 family has only been initially studied yet (9, 10). hSGLT5 is expressed almost exclusively in the kidney cortex, but its role in sugar homeostasis has not been completely resolved. A recent initial characterization of hSGLT5 expressed in T-Rex HEK293 cells has shown that it is a Na\(^{+}\)-dependent, phlorizin-sensitive hexose transporter with an unusual sugar selectivity (9). It has a high affinity for D-mannose and D-fructose (\(K_{0.5} \approx 1\) mM, relative to that for D-galactose and \([^{14}C]H_9251\)-MDG, \(K_{0.5} \approx 10\) mM), more similar to hSGLT4 than either hSGLT1 or hSGLT2 (28). These data (9) suggest that hSGLT5 is probably involved in the control of plasma mannose levels by regulating its reabsorption in the kidney. In this study we have examined the functional characteristics of hSGLT5 in detail using HEK293-T-Rex cells, radioactive transport assays, and whole cell patch-clamp electrophysiology similar to that used previously for hSGLT1 and hSGLT2 (15).

We found that Na\(^{+}\)/mannose cotransport by hSGLT5 is electrogenic. Superfusing cells expressing hSGLT5 with mannose in a NaCl buffer generated an inward current that was abolished by phlorizin (Fig. 4). These currents were not observed when Na\(^{+}\) was replaced by choline, indicating that Na\(^{+}\) is the charge carrier. Sodium is the preferred cation to drive mannose translocation, but as in SGLT1 (13), two other cations, i.e., lithium and protons, can partially substitute for sodium (Fig. 2) with protons being the best substitute. The apparent affinity for sodium was \(\sim 35\) mM, similar to the values measured for hSGLT1 and hSGLT2 (9). Interestingly
Na⁺ activation kinetics (Fig. 2B) showed that the Na⁺-dependent mannose transport had a Hill coefficient of 1, suggesting a 1:1 Na⁺:mannose ratio as observed for hSGLT2 (15). A definitive conclusion about the stoichiometry of hSGLT5 will require a more detailed analysis, and we suspect that the single Na⁺ binds to the Na2 site conserved in all the members of the SLC5 family (19).

We examined the sugar selectivity of hSGLT5 using competition studies. [¹⁴C]mannose (50 μM) uptake was inhibited completely by adding 100 mM d-mannose to the extracellular solution (Fig. 3), as expected from the mannose Kᵦₛ, 0.45 mM (9). On the other hand, ribose inhibited 30%, xylose 10%, and lyxose 50%, indicating that the Kᵦₛ for xylose and lyxose and ribose is higher than 100 mM (Fig. 3B). Previous studies have also shown that removal of the equatorial/axial orientation of the hydroxyls has a large effect on the sugar apparent affinity of hSGLT1 by a factor of 5 to >200. In particular, replacing the equatorial 2-hydroxyl group with hydrogen (2-DOG) results in the reduction of the apparent affinity by ~100 fold, and placing this hydroxyl in the axial orientation (mannose) resulted in the lack of interaction. Similar experiments with hSGLT5 show a 20-fold loss of binding of 2-DOG to hSGLT5 (Fig. 3C). The hexose mannose is preferred over the pentose homomorph n-lyxose and an axial –OH group on C-2 provides a kinetic advantage for mannose over d-glucose and 2-DOG (Fig. 3). Moreover, no significant inward currents were produced by 10 mM  α-MDG, confirming the substrate specificity of hGLT5.

Table 1 summarizes the kinetic properties of human SGLT isoforms expressed in mammalian cells. Both SGLT4 and SGLT5 prefer mannose as a substrate over glucose, while SGLT1 and SGLT2 prefer glucose over mannose. Galactose is a substrate for SGLT1 and SGLT5 but not SGLT2 or SGLT4. SGLT5 appears to be unique in its ability to transport fructose. The sugar binding site has been identified in the crystal structure of a bacterial homolog of the mammalian SGLTs, Vibrio parahaemolyticus vSGLT (6), and shows that the three-dimensional structure of the coordinating residues, are generally conserved in the SGLT family (28), e.g., N78, H83, E102, Y290, W291, K321, and Q457 in hSGLT1 (22). In hSGLT5 all are conserved except N78S and H83L; interestingly, histidine 83 is substituted with a leucine in hSGLT4, as for hSGLT5.
suggesting a possible role of this residue in determining the substrate specificity of the protein. Are these positions sufficient to account for the differences in selectivity between SGLT1 and SGLT5? Probably not as mutation of histidine 83 to cysteine (H83C) in hSGLT1 does not enable the protein to transport mannose, fructose, or 2-DOG (unpublished data, Gorraitz E). Further studies would be required to draw a final conclusion but most probably more residues are involved in determine substrate interaction and specificity.

As different studies (7) indicate a major role of hSGLT5 in the regulation of plasma mannose concentration, we decided to investigate the localization of hSGLT5 and its physiological role in vivo. To determine if hSGLT5 is expressed in the kidney, we decided to measure [14C]mannose and [14C]-MDG uptake in rat kidney slices. As shown in Fig. 5, we did not detect any phlorizin sensitive mannose uptake comparable to that for α-MDG.

In contrast, the SGLT5 knockout mouse model (7) showed that Na+-dependent fructose and mannose uptakes into renal brush border membrane vesicles were reduced relative to those from wild-type mice. This points to a physiological role of SGLT5 in the reabsorption of fructose, and perhaps mannose, from the glomerular filtrate. The importance of SGLT5 in glucose reabsorption was not reported.

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DISCLOSURES
E. M. Wright serves on a SGLT2 Inhibitor Advisory Board for Boehringer Ingelheim; has a consultant for BMS/AZ, Roche, Merk, and Novartis on SGLT biology; and has been a speaker on SGLT biology at industry-sponsored symposia and workshops at national society meetings.

AUTHOR CONTRIBUTIONS
Author contributions: C.G., E.G., and E.M.W. conception and design of research; C.G. and E.G. performed experiments; C.G. and E.G. analyzed data; C.G., E.M., and E.M.W. approved final version of manuscript.

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