Sugar Binding to Na\textsuperscript{+}/Glucose Cotransporters Is Determined by the Carboxyl-terminal Half of the Protein*

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\(\text{D-Glucose is absorbed across the proximal tubule of the kidney by two Na}^{+}/\text{glucose cotransporters (SGLT1 and SGLT2). The low affinity SGLT2 is expressed in the S1 and S2 segments, has a Na}^{+}/\text{glucose coupling ratio of 1, a } K_{0.5} \text{ for sugar of } 2 \text{ mM, and a } K_{0.5} \text{ for Na}^{+} \text{ of } 1 \text{ mM. The high affinity SGLT1, found in the S3 segment, has a coupling ratio of 2, and } K_{0.5} \text{ for sugar and Na}^{+} \text{ of } 0.1 \text{ and } 0.2 \text{ mM, respectively. We have constructed a chimeric protein consisting of amino acids 1-380 of porcine SGLT2 and amino acids 381-662 of porcine SGLT1. The chimera was expressed in Xenopus oocytes, and steady-state kinetics were characterized by a two-electrode voltage-clamp. The } K_{0.5} \text{ for } \alpha\text{-methyl-D-glucopyranoside (0.2 mM) was similar to that for SGLT1, and like SGLT1 the chimera transported } \text{D-galactose and 3-O-methylglucose. In contrast, SGLT2 transports poorly } \text{D-galactose and excludes 3-O-methylglucose. The apparent } K_{0.5} \text{ was 3.5 mM (at } 150 \text{ mM), and the Hill coefficient ranged between 0.8 and 1.5. We conclude that recognition/transport of organic substrate is mediated by interactions distal to amino acid 380, while cation binding is determined by interactions arising from the amino- and carboxy-terminal halves of the transporters. Surprisingly, the chimera transported } \alpha\text{-phenyl derivatives of } \text{D-glucose as well as the inhibitors of sugar transport: phlorizin, deoxynphlorizin, and } \beta\text{-glucopyranosylphenyl isothiocyanate are transported with high affinity (} K_{0.5} \text{ for phlorizin was 5 } \mu\text{M). Thus, the pocket for organic substrate binding is increased from } 10 \times 5 \times 5 (\tilde{A}) \text{ for SGLT1 to } 11 \times 18 \times 5 (\tilde{A}) \text{ for the chimera.}

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\footnote{The abbreviations used are: SGLT1 (SGLT2), high (low) Na\textsuperscript{+}/glucose transporter for D-glucose; aMDG, a-\text{methyl-D-glucopyranoside; } a\text{-PG, phenyl- }\alpha\text{-D-glucopyranoside; } a\text{-PG, phenyl- }\beta\text{-D-glucopyranoside; 3-O-Me-Glc, 3-O-methyl- }\beta\text{-D-glucopyranoside; } \beta\text{-Nap-Glc, } \beta\text{-naphthyl }\beta\text{-D-glucopyranoside; phloretin, } [3\text{-[4-hydroxypyrenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone; Pz, phlorizin, phloretin-2'- }\beta\text{-glucoside; arbutin, 4-hydroxyphenyl- }\beta\text{-D-glucopyranoside; GPITC, } \beta\text{-glucopyranosylphenyl isothiocyanate.}}

\footnote{The product of this three-fragment ligation was a chimeric sequence, coding for amino acids 1-380 from pig SGLT2 and amino acids 381-662 from porcine SGLT1, and amino acids 381-662 (which form putative transmembrane helices 1-8 of pSGLT2, and a } K_{0.5} \text{ for sugar of 6 mM and a } K_{0.5} \text{ for Na}^{+} \text{ coupling ratio of 1. The reabsorption process is completed in the S3 segment by another Na}^{+}/\text{glucose cotransporter (SGLT1) with significantly higher apparent affinity for sugar (} K_{0.5} \text{ for SGLT1} \text{ than SGLT2 and a Na}^{+}/\text{glucose coupling ratio of 2.}

\footnote{Recently a low affinity Na\textsuperscript{+}/glucose cotransporter (pSGLT2) from LLC-PK1 cells was cloned and functionally characterized (17, 18). While the amino acid sequence of pSGLT2 was highly homologous to pSGLT1 (75% identical; 88% similar amino acid residues), the apparent } K_{0.5} \text{ for aMDG was } 2 \text{ mM, an apparent } K_{0.5} \text{ of 2-7 mM, and a Hill coefficient } >1.5.

\footnote{Provided this high similarity of the primary sequences and secondary structure of SGLT1 and SGLT2, what accounts for these differences in functional properties? Which parts of the proteins are involved in Na\textsuperscript{+} and sugar binding? To begin to answer these important questions, we generated a chimeric DNA-construct corresponding to amino acids 1-380, which form the putative transmembrane helices 1-8 of pSGLT2, and amino acids 381-662 (which form putative helices 9-14 of pSGLT1) and compared its functionality with the parental proteins.}

\footnote{EXPERIMENTAL PROCEDURES

Molecular Biology Methods—A SpeI/Stul fragment from pig pSGLT2 in pBluescript SK\textsuperscript{+} (clone pCTK; Ref. 17) was ligated together with a Stul/XhoI fragment from pig ionpSGLT1 (derived from donor pPGST-B1; Ref. 10) and a SpeI/XhoI fragment from pMAMneo-SGLT1 plasmid (20). The product of this three-fragment ligation was a chimeric sequence, coding for amino acids 1-380 from pig SGLT2 and amino acids 381-662 from pig SGLT1 (2.4-kilobase BamHI/XhoI coding fragment). To provide the chimeric DNA with the essential viral promoter sequence for in vitro transcription, and a poly(A) tail for expression in oocytes, the BamHI and XhoI 3'-ends were filled in by using Klenow enzyme. After purification by agarose gel electrophoresis, it was ligated into the Smal site of the polylinker region of vector pAK52 (18). This template DNA was linearized with EcoRI and transcribed in vitro and capped with SP6 RNA polymerase by using the Ambion MEGAscript transcription kit (Ambion, Austin, TX), and capped with SP6 RNA polymerase by using the Ambion MEGAscript transcription kit (Ambion, Austin, TX).}
Oocyte Maintenance and Functional Assays—Mature Xenopus oocytes were manipulated as described previously (21). Na⁺-dependent \(\alpha\)MDG or phlorizin influxes were measured by a standard procedure (22), where groups of 7–10 control or cRNA-injected oocytes were incubated for 1 h in 5 \(\mu\)M \(^3\)Hphlorizin (specific activity 47.6 Ci/mmol; DuPont NEN) or 50 \(\mu\)L [\(3^C\)]aMDG (specific activity 293 mCi/mmol; Amersham).

All two-microelectrode voltage clamp studies were controlled by the Clampex computer program of pCLAMP software (Axon Instruments, Foster City, CA) as described in Ref. 12. During experiments measuring steady-state kinetics, the oocyte membrane voltage was first held at \(V_h = -50 \text{ mV}\). Then step changes to 11 different test potentials (\(V_t\) in 20-mV intervals between -50 and -150 mV (each of a duration of 100 ms) were applied. The voltage pulse to the test potential was followed by return of the membrane voltage to the holding potential. Currents were averaged over three sweeps and low-pass-filtered at 500 Hz by an eight-pole Bessel filter.

Sugar-induced steady-state currents at different substrate concentrations (S) were fitted to Equation 1 by nonlinear regression analysis using Sigma Plot (Systat, San Rafael, CA).

\[
I = \frac{I_{\text{max}}(S)}{I_{\text{max}} + [S]^n}
\]

\(I\) represents the substrate (sugar or cation)-induced current, \(I_{\text{max}}\) is the calculated maximal current, \(n\) is the coupling coefficient, and \(K_{0.5}\) is the substrate concentration for 50% \(I_{\text{max}}\).

Sugar and phenylglucosides were purchased from Sigma. Three-dimensional images of these were created by computational modeling based on the program Hyperchem 2.0 (Autodesk, Sausalito, CA) as described in Ref. 13.

Multiple sequence alignments of the amino acid sequences were performed using the program PILEUP (Genetics Computer Group, Madison, WI).

RESULTS

Sugar- and Phlorizin-induced Steady-state Currents—Fig. 1A shows the current records for the chimera after the membrane voltage was stepped to various test values \(V_t\) (+50, +30, -10, -110, and -150 mV) from the holding voltage \(V_h = -50 \text{ mV}\). The left panel shows the currents in the absence of sugar in NaCl buffer, and the right panel shows the currents after 20 mM aMDG was added to the bath solution. The current relaxation consisted of an initial capacitive transient (time constant \(-0.5\)–1 ms), followed by a slower decay (time constant \(-11\) ms, at \(+50 \text{ mV}\)) to the steady-state. This slow component, most pronounced at the depolarizing potentials (Fig. 1A, left panel), is the pre-steady-state current of the chimera.

The sugar-induced current is the difference in the steady-state currents in the presence and absence of sugar. Addition of the saturating concentration of aMDG to the bath solution evoked an increase of the inward current of \(-137 \text{ nA at -150 mV}\) and completely eliminated the pre-steady-state current (Fig. 1A, right panel). The right panel of Fig. 1B shows the aMDG-induced current, measured on another oocyte, as a function of voltage. The current increased as the membrane potential increased from -50 to -150 mV, but did not reach saturation at the most negative voltage applied. The steady-state current at \(-150 \text{ mV}\) was \(-89 \text{ nA}\).

When the choline chloride solution was replaced by NaCl, the chimera generated an inward current in the absence of sugar (Fig. 1B). This sugar-independent current, or Na⁺-leak, has also been observed for SGLT1 (24) and SGLT2 (18). The sugar-independent Na⁺ current of the parent proteins can be estimated either as the difference in the currents when external Na⁺ is replaced by choline (Fig. 1B, left panel) or as the current blocked by phlorizin in the absence of sugar. Phlorizin has been shown to block the sugar-independent current in SGLT1 (24, 14) and SGLT2 (18). Surprisingly, when phlorizin was added to the 100 mM NaCl buffer in the absence of sugar (on the same oocyte presented in Fig. 1B), there was an increase in inward current instead of a block (Fig. 1C, left panel). The current-voltage relationship of the current induced by 100 \(\mu\)M phlorizin is shown in the right panel of Fig. 1C and was similar in amplitude \((-108 \text{ nA at -150 mV}\) as well as in voltage dependence to the current induced by 20 mM aMDG (Fig. 1B, right panel).

For the chimera, the ratio between the sugar-independent current (estimated by choline substitution as described above) and the sugar (aMDG)-mediated current was considerably higher than observed for both SGLT1 and SGLT2. Both parental transporters show a sugar-independent current (the Na⁺-leak current) that is \(-10\%\) of the sugar-mediated current (15, 18). In contrast, the Na⁺-leak current mediated by the chimera was relatively higher, 44% of the maximal current induced by 20 mM aMDG. Similar values were obtained in four additional experiments.

To confirm that the phlorizin- or aMDG-induced currents were accompanied by uptake of phlorizin or aMDG into the oocytes, we measured the uptake of radiolabeled phlorizin or sugar in 100 mM NaCl or 100 mM choline chloride. Fig. 2 shows
that oocytes expressing the chimera transported eight times more \[^{14}C\]MDG and \[^{3}H\]phlorizin in 100 mM NaCl than non-injected oocytes. No phlorizin transport could be measured in SGLT1 cRNA injected oocytes, and 100 mM choline chloride did not support any uptake of phlorizin into oocytes injected with chimeric cRNA (not shown).

The current induced by \( \alpha \)MDG was strongly dependent on membrane voltage and increased with more negative test voltages. Fig. 3A shows that the current-voltage relationship at 20 mM \( \alpha \)MDG did not saturate in the voltage range from \(+50\) to \(-150\) mV and reached values in different oocytes between \(-80\) and \(-300\) nA (at \(-150\) mV). To obtain the apparent affinity constant for \( \alpha \)MDG (\( K_{\alpha,5} \)) in 100 mM NaCl, currents induced by different sugar concentrations were fitted to Equation 1. The obtained \( K_{\alpha,5} \) of 0.25 mM was close to the value for the high affinity transporter SGLT1 and voltage independent (Fig. 3B). Dependent on the level of expression the maximal sugar-induced current \( I_{\alpha,\text{max}} \) was between 90 and 250 nA (at \(-150\) mV).

To obtain the kinetic description of phlorizin transport in 100 mM Na\(^+\), the phlorizin-induced currents were measured as a function of different external phlorizin concentrations (0–100 \( \mu \)M). Similar to the I/V curve of the current induced by sugar (\( \alpha \)MDG), the I/V curve of the current induced by phlorizin did not saturate in the voltage range from \(+50\) mV to \(-150\) mV (Fig. 1C, right panel). \( I_{p,\text{max}} \) reached between \(-100\) and \(-300\) nA (at \(-150\) mV) and was comparable to the \( I_{\alpha,\text{max}} \). The apparent \( K_{\alpha,5} \) for phlorizin transport (\( K_{p,5} \)) in 100 mM NaCl obtained from two experiments was \( 4.5 \pm 0.8\) \mu M and voltage independent (not shown).

Substrate Specificity—To determine the substrate specificity, we measured the steady-state currents induced by 20 mM concentrations of various substrates at \(-150\) mV. For comparison between different substrates and among oocytes, the currents were normalized to the current mediated by 20 mM D-glucose, a saturating sugar concentration (Fig. 4). The substrates were divided into glucose analogues and phenylglucosides. The chimera showed highest affinity to D-glucose and \( \alpha \)MDG and moderate affinity for D-galactose and 3-O-methylglucose. L-Glucose, 2-deoxyglucose, and mannose are nontrans-
DISCUSSION

To date, little information is available on domains or residues responsible for ion and organic substrate recognition and binding in the family of Na⁺/glucose cotransporters. In this study we show that functional chimeras can be obtained between homologous members of this family. We expressed a protein which according to the predicted topology for SGLT (19) consisted of putative membrane helices 1–8 of SGLT2 and putative membrane helices 9–14 of SGLT1.

KMDG and Substrate Specificity—The KMDG for the chimera of 0.2 mM and its voltage independence, as well as the specificity for the glucose analogues, D-galactose and 3-O-methylglucose, closely resembled those of the high affinity SGLT1 (see Fig. 3B and Fig. 4). In SGLT1, the affinity for D-galactose (KMDG = 0.2 mM; Ref. 27) is similar to that for D-glucose, while the affinity for 3-O-methylglucose is lower (KMDG = 2–8 mM; Ref. 16). For SGLT2, D-galactose is a very poor substrate (it induces only 5% of the maximal glucose-induced current) with KMDG > 20 mM (18) and 3-O-methylglucose is not transported at all. The chimera transported D-galactose and 3-O-methylglucose with affinities which were similar to SGLT1 (the induced currents were +60% and 36% of the glucose-induced currents, respectively). Thus, the predominant phenotype of the chimera was dearly close to the high affinity transporters phenotype. Since the carbonyl-terminal half of the chimera was delivered by SGLT1, this indicates that the residues of the carbonyl-terminal half of the SGLT proteins determine the high affinity transport of sugar. This observation is similar to the galactose and glucose (Gal2) facilitated transporter from yeast, where the substrate recognition domain is in 101 amino acids close to the carbonyl terminus (21). For the rat facilitated glucose transporter (GLUT1), the region encompassing membrane spans 7–12 was observed to have a crucial role in glucose transport (29), and Due et al. (30) specified that the last 29 amino acids in the carbonyl-terminal tail are the important residues in determining the affinity to the substrate.

The phenylglucoside phlorizin is the most potent inhibitor of both SGLT1 and SGLT2, but it was transported by the chimera with high affinity. Dependent on the pH, the conformation of the aglucone of phlorizin (phloretin) varies between two tautomeric (keto-enol) forms. At pH 5.5, the absorption peak at 285 nm corresponds to the keto form, and, at pH 8.4, the maximal absorption at 320 nm is due to the enol form (31). Between pH 5.5 and 8.5, we observed that maximal transport of phlorizin

![Figure 5. Na⁺ activation of the steady-state current.](image)

The Na⁺ concentration of the bath solution [Na⁺], varied between 0 and 100 mM (0, 0.5, 1, 5, 10, 20, 50, 70, and 100), whereas [αMDG] was maintained at 20 mM. Shown is the dependence of the steady-state current at −150 mV with increasing [Na⁺]. The curve is drawn according to the fit.

estimates of $K_{MDG}^a$ and $n$ for Na⁺ because of the large parameter errors.
of specific interactions with the naphthyl moiety, rather than by a steric hindrance due to the bulky aglucone. In addition, since the chimera does not transport arbutin (see structural formulas in Fig. 6B), we speculate that in this case there is a lack of electrostatic interactions in the area surrounding the para-position of the phenyl ring.

Affinity for Cations by the Chimera—Since in both parental proteins, sugar transport is supported by Na\(^+\), Li\(^+\), and H\(^+\) (33, 34) and is similar to that observed in the chimera, no conclusions can be drawn on the location of the cation-recognizing domains by this construct. The parental proteins showed a major difference in their Hill coefficient for Na\(^+\): n was 1 for SGLT2 and 2 for SGLT1 (17), and both were voltage independent. Because of the low amplitude of the sugar-evoked currents at low Na\(^+\) concentrations, the determination of the kinetic parameters \(K_{Na}^0\) and (especially) the Hill coefficient of the chimera became problematic. These parameters could not be estimated accurately and related to the low or high affinity transporters. Nevertheless, the chimera still possessed a high affinity for Na\(^+\) ions (~3.5 mM at ~150 mV). Analysis of new chimeras which combine the amino-terminal half of SGLT1 and the carboxyl-terminal half of SGLT2 could be successful in resolving the structural code for Na\(^+\) binding.

Comparison with Other Structure-Activity Studies on SGLT—The major conclusion of the present study is that the carboxyl termini of the SGLT family members modulate the selectivity and affinity for sugar substrates. This statement agrees with previous structure-function studies on site-directed mutants of SGLT1. Substitution of aspartic acid at position 176 (Asp-176) with alanine, influenced only the pre-steady-state kinetics and not the sugar-induced steady-state currents or the \(K_{Na}^0\) (21). Replacement of a conservative lysine residue at position 321 (Lys-321) with alanine decreased dramatically the affinity for Na\(^+\) ions, while having secondary effects on sugar binding to the protein (35). Both residues are in the amino-terminal half of SGLT1 and seem not substantially to affect sugar binding. In contrast, a missense mutation (R499H) from a patient with glucose/galactose malabsorption, localized close to the carboxyl terminus of the protein (36), decreased the apparent affinity for sugar to human SGLT1 (\(K_{Na}^0\) raised to 2 mM), without affecting the apparent K\(_{Na}^0\) of SGLT2 and 2 for SGLT1 (17), and both were voltage independent. Because of the low amplitude of the sugar-evoked currents at low Na\(^+\) concentrations, the determination of the kinetic parameters \(K_{Na}^0\) and (especially) the Hill coefficient of the chimera became problematic. These parameters could not be estimated accurately and related to the low or high affinity transporters. Nevertheless, the chimera still possessed a high affinity for Na\(^+\) ions (~3.5 mM at ~150 mV). Analysis of new chimeras which combine the amino-terminal half of SGLT1 and the carboxyl-terminal half of SGLT2 could be successful in resolving the structural code for Na\(^+\) binding.

Comparison of the pre-steady-state (12) and the steady-state (16) kinetic parameters of the SGLT1s from rat, rabbit, and human have given initial ideas about residues that may account for the functional differences between these isoforms. Since the amino terminus (residues 1–27) and two hydrophilic loops located in the center (residues 229–271) and the carboxyl terminus (residues 548–644) involve most of the nonconserved polar residues between the three species, it has been proposed that they possibly contribute for charge movement and/or substrate specificity differences. The present study locates the sugar recognition domain distal to amino acid 380. Therefore, a comparison of the primary sequences distal to amino acids 380 of all cloned high affinity transporters from rat (11), human (37), rabbit (38), and porcine (10), with the corresponding sequence of the low affinity transporter from porcine (17, 18), should specify individual amino acids that modulate sugar recognition (Fig. 7). According to the recently proposed topology for SGLT (19) and taking conservative substitution into account (K = R, S = T, D = E, Y = F = W, and I = V = L = M), our comparison localized differences in the loops between M10/ M11, M12/M13, and M13/M14. In particular, in the loop M10/ M11, two conserved serines (Ser-446, Ser-449) in the high affinity subfamily were substituted in SGLT2 by Val-446 and Asn-449; the conservative aspartic acid Asp-455 was substituted by a countercharge in SGLT2 (His-455), and, in the loop

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2 B. A. Hirayama, personal communication.
3 B. Mackenzie, manuscript in preparation.

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**Fig. 6. Superimposition of predicted three-dimensional structures of some phenylglycosides.** The three-dimensional images of phenylglycosides were obtained by conformational search and energy minimizations as described in Ref. 23. A, transported substrates by the chimera. Presented are the β-phenyl glycosides phlorizin, GPITC, and the α-phenyl glycoside (α-PG). The phenyl ring of β-phenyl glycoside (β-PG) superimposes with the phenyl ring of GPITC and is not explicitly indicated in the figure. B, transported substrates by the high-affinity SGLT1 isoforms. Arbutin is transported by all three SGLT1 isoforms from rat, rabbit, and human, whereas β-Naph-Glc is transported by the rat and human isoforms, but not by rabbit SGLT1 (16).

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The superimposed three-dimensional geometry of the phenylglycoside substrates of the chimera (Fig. 6, upper panel) indicated outer dimensions of 11 × 18 × 5 (Å). The transport pocket for substrate in the case of the high affinity (SGLT1) human and rat isoforms is significantly smaller: 10 × 5 × 5 (Å), and the bulky known transported substrate is β-Naph-Glc (Fig. 6B). The rabbit SGLT1 isofrom does not transport this substrate (16). Thus, we conclude that the chimera provides a less restrictive selectivity, compared to SGLT1 (comparable data are not yet available for SGLT2). In view of the transport of phlorizin, deoxyphlorizin, and GPITC by the chimera, its inability to transport β-Naph-Glc is probably caused by the loss of the ability to transport the keto form.2
between M12/M13, two adjacent conservative residues Glu-514 and Pro-515 were both replaced in SGLT2 by a neutral alanine (Ala-514, Ala-515). The largest loop between M13/M14 contained most of the substitutions: a conservative serine (Ser-562) in the SGLT1s was replaced by an alanine (Ala-562) in SGLT2; and both conserved acidic residues (Glu-577) and aspartic acid (Asp-614) were missed in SGLT2. In the same loop, aspartic acid (Asp-580), isoleucine (Ile-581), and glutamine (Gln-582) were all replaced by positively charged residues in SGLT2: lysine (Lys-580), arginine (Arg-581), and histidine (His-582). A lysine (Lys-599) was converted into a threonine (Thr-599) in SGLT2, a methionine (Met-629) to glutamine (Gln-629) and leucine (Leu-631) to an arginine (Arg-631). Any of these amino acids could contribute to the organic substrate binding. This easily could be tested by site-directed mutagenesis.

The presented structure-functional analysis of a chimeric protein between two members of the SGLT family initiates a powerful strategy for further studies in localizing the structural determinants for cotransporter function.

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Fig. 7. Amino acid sequence alignment of the SGLT family members. Shown are the sequences from the carboxyl-terminal halves of the high affinity cotransporters (SGLT1s) from porcine, human, rat, mouse, ovine, rabbit, and the low affinity cotransporter (SGLT2) from porcine. The dashes (---) represent identical residues and conservative substitutions (see text), and the shaded regions are putative membrane domains (19).
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