Neutralization of a Conserved Amino Acid Residue in the Human Na\(^+\)/Glucose Transporter (hSGLT1) Generates a Glucose-gated H\(^+\) Channel

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The role of conserved Asp\(^{204}\) in the human high affinity Na\(^+\)/glucose cotransporter (hSGLT1) was investigated by site-directed mutagenesis combined with functional assays exploiting the Xenopus oocyte expression system. Substitution of H\(^+\) for Na\(^+\) reduces the apparent affinity of hSGLT1 for glucose from 0.3 to 6 mM. The apparent affinity for H\(^+\) (7 \(\mu\)M) is about three orders of magnitude higher than for Na\(^+\) (6 mM). Cation/glucose cotransport exhibits a coupling ratio of 2 Na\(^+\) (or 2 H\(^+\)):\(\text{glucose}\). Pre-steady-state kinetics indicate that similar Na\(^+\)- or H\(^+\)-induced conformational changes are the basis for coupled transport. Replacing Asp\(^{204}\) with Glu increases the apparent affinity for H\(^+\) by >20-fold with little impact on the apparent Na\(^+\) affinity. This implies that the length of the carboxylate side chain is critical for cation selectivity. Neutralization of Asp\(^{204}\) (Asp \(\rightarrow\) Asn or Cys) reveals glucose-evoked H\(^+\) currents that were one order of magnitude greater than Na\(^+\) currents. These chlorozin-sensitive H\(^+\) currents reverse and are enhanced by internal acidification of oocytes. Together with a H\(^+\) to sugar stoichiometry as high as 145:1, these results favor a glucose-gated H\(^+\) channel activity of the mutant. Our observations support the idea that cotransporters and channels share common features.

The human high affinity Na\(^+\)/glucose cotransporter (hSGLT1) is a member of a family of secondary transporter proteins encompassing more than 55 homologues from archaea, bacteria, yeast, insects, and mammals (1, 2). This family uses gradients to drive the coupled uphill transport of a variety of substrates (sugars, amino acids, vitamins, osmolytes, ions, myo-inositol, urea, and water). The expression of hSGLT1 in Xenopus laevis oocytes has resulted in a comprehensive study of both steady-state and pre-steady-state kinetics (3–5). A six-state ordered binding model has been proposed in which transport results from ligand-induced conformational changes (6, 7). In this model Na\(^+\) binds before sugar, with a coupling ratio of 2 Na\(^+\) : 1 glucose, and voltage influences both Na\(^+\) binding and the conformational states of the unloaded transporter. Functional analysis of SGLT chimeras and truncated proteins strongly suggests that the sugar pathway is located in the C-terminal domain of the protein (8, 9). Site-directed thiol labeling of a residue in the proposed sugar pathway indicates that conformational changes are responsible for the coupling of Na\(^+\) and sugar transport (10). We suggest that these conformational alterations are induced by cation binding in the N-terminal domain of the protein.

Although the functional importance of the N terminus in cation binding/translocation was shown for another SGLT family member (the Na\(^+\)/proline transporter (PutP) of Escherichia coli) (11–13), there is little information on the role of the N-terminal domain in hSGLT1. We have initiated a study to explore the role of N-terminal residues in hSGLT1 in cotransport. In the present study, we have targeted a conserved residue, Asp\(^{204}\), located in a short cytoplasmic loop of hSGLT1 connecting transmembrane domains V and VI, which has been implicated in cation selectivity in PutP (12). Replacing Asp\(^{204}\) in hSGLT1 with Asn, Cys, or Glu dramatically modulated the steady-state and pre-steady-state kinetics of the transporter. Remarkably, although a transporter with a negative amino acid (Asp or Glu) exhibited cation/glucose cotransport with a stoichiometry of 2 (Na\(^+\) or H\(^+\)) : 1, neutralization of Asp\(^{204}\) (by Asn or Cys) resulted in the activation of a glucose-activated H\(^+\) channel.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—A plasmid containing human SGLT1 (hSGLT1) cDNA was used as template for site-directed mutagenesis. Replacement of Asp\(^{204}\) with Asn, Cys, and Glu was performed using a two-step polymerase chain reaction protocol (14). For each pair of mutagenic oligonucleotides the sequence of the sense primer is presented with the altered nucleotide(s) underlined: D204A, 5′-GATTTACAGTGCACCTTGGC-3′; D204E, 5′-GATTTACGGAACCTTGGC-3′; D204N, 5′-GATTTACGGAGACCTTGGC-3′. Polymerase chain reaction products were digested with BglII and Eco47III, and the resulting 428-bp fragments were ligated into a similarly treated wild-type hSGLT1-containing plasmid. The fidelity of the inserted DNA fragments was confirmed by sequencing double-stranded DNA (Sequenase version 2.0, DNA sequencing kit, United States Biochemical, Cleveland, OH) after alkaline denaturation (15). Each mutagenized DNA template was linearized with XbaI, transcribed, and capped in vitro using the T3 RNA promoter (MEGAscript kit, Ambion, Austin, TX). X. laevis oocytes were injected with 50 ng of mRNA and were incubated in Barth medium containing gentamicin (5 mg/ml) at 18 °C for 3–7 days (4).

Transport Assays and Electrophysiological Techniques—For transport and electrophysiological experiments, oocytes were bathed in an assay buffer composed of 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES-Tris, pH 7.5, and a combination of Na\(^+\) or choline chloride salts to give a final concentration of 100 mM. For \(H^+\) activation experiments the pH of 100 mM choline buffer was varied between 8.0 and 4.5 by titration with Tris or Mops. Uptake of methyl-[\(^{14}\)C]glucopyranoside (293 Ci/mol, Amersham Pharmacia Biotech) and electrophysiological measurements using the two-microelectrode voltage clamp technique were performed as described (16, 17). The stoichiometry of

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The abbreviations used are: hSGLT1, human high affinity Na\(^+\)/glucose cotransporter; oMDG, α-methyl-d-glucopyranoside; Glc, α-glucose; H\(^+\), 3.2 \(\mu\)M H\(^+\); pH 5.5; Na\(^+\), 100 mM Na\(^+\); WT, hSGLT1-wild-type; PutP, Na\(^+\)/proline transporter; Mes, 4-morpholineethanesulfonic acid; nC, nanocoulomb(s).
cation-coupled D-[U-14C]glucose (316 Ci/mol, ICN Radiochemicals) uptake was determined under voltage clamp conditions (18).

Data Analysis—Sugar-evoked steady-state currents were fitted to Eq. 1,

$$f^{\text{max}} = \frac{f^{\text{max}} \times [\text{Glc}]}{K_{\text{app}} + [\text{Glc}]}$$  \hspace{1cm} (Eq. 1)

where $f^{\text{max}}$ and $f^{\text{max}}$ represent cation-glucose-induced current and maximal cation-glucose-induced current, respectively, at saturating cation ([C]), $K_{\text{app}}$ is [C] at 0.5 $f^{\text{max}}$, and $z$ represents the Hill coefficient. Charge-voltage ($Q$-$V$) relations for each membrane voltage ($V_m$) were obtained by integrating pre-steady-state current transitions (after subtracting the capacitive and the steady-state currents from the total currents) with $Q_{\text{app}}$ and $Q_{\text{exp}}$ for $Q$ at hyperpolarizing and depolarizing limits, respectively. $V_{50}$ represents $V_m$ at which 50% of the total charge in the membrane electric field has moved, $z$ is the apparent valence of the mobile charge, and $F$, $R$, and $T$ have their usual meanings. All experiments were repeated at least three times with oocytes from different donor frogs. Data fits were performed using the non-linear regression algorithm in SigmaPlot (version 5.0, SPSS Inc., Chicago, IL). Unless otherwise noted, figures are based on data obtained from a typical experiment on a single oocyte, and errors represent S.E. of the fit.

RESULTS

In these studies all comparisons between Na+ and H+ kinetics were performed in the same oocyte expressing WT, D204C, D204E, or D204N cRNA. For comparison of WT kinetics and the kinetics of a transporter with a substitution at position 204, oocytes of the same batch were analyzed on the same day within 10 h.

Uptake Experiments—The most conservative substitution Asp204 $\rightarrow$ Glu resulted in a reduction of Na+ dependent DMDG uptake by 87%, whereas the uptake rates of D204C and D204N were reduced by $\sim$50% (Fig. 1). In the absence of Na+ ions, the rate of DMDG uptake in oocytes expressing D204E was about 1 pmol/h, a value comparable to oocytes with the native transporter (1.7 pmol/h $\times$ h$^{-1}$ $\times$ oocyte$^{-1}$). However, replacing Asp204 with Cys or Aas increased the uptake rate by 7- and 16-fold.

This increase may reflect the enhanced apparent affinity for sugar (see Table I).

Steady-State Currents—Fig. 2 shows representative sugar-induced current-voltage relationships for WT, D204C, and D204E in the presence of NaCl or HCl at saturating [Glc]. For WT both curves were sigmoidal and saturated at hyperpolarizing voltages. A similar I-V relation was observed for D204E in NaCl, but in HCl the sugar-induced currents exhibited a supralinear increase with hyperpolarization. D204C and D204N (data not shown) exhibited essentially identical I-V relations with no saturation of the sugar-evoked currents at the most negative potential ($\sim$150 mV) in either NaCl or HCl. The glucose-evoked currents in HCl for these transporters were much greater than the NaCl (see Table I). In HCl for the latter transporters, glucose activated outward currents at potentials more positive than $+20$ mV ($+10$ nA at +50 mV). A comparison of the sugar-induced currents at $V_m$ = $-150$ mV revealed similar values in the presence of HCl for WT ($\sim$1220 nA), D204C ($\sim$1120 nA), and D204N ($\sim$870 nA), and a reduction of about 75% for D204E ($\sim$300 nA). In NaCl, WT and D204E showed comparable currents generated by 100 mM glucose at $-150$ mV ($\sim$1480 and $-930$ nA). Under the same test conditions, D204C and D204N exhibited less than 10% of the current observed for WT.

We measured the sugar kinetics of each transporter by varying external [Glc] from 0.05 to 100 mM in the presence of NaCl or HCl. The apparent affinity constants for D-glucose ($K_{\text{Glc}}^{\text{app}}$ at $V_m$ = $-110$ mV are shown in Table I. As described earlier (20), hSGLT1 exhibited a $K_{\text{Glc}}^{\text{app}}$ of 0.3 mM at NaCl. When HCl substituted for NaCl, the apparent affinity for Glc was reduced by 20-fold ($K_{\text{Glc}}^{\text{app}}$ = 6 mM). A similar reduction of the apparent Glc affinity in HCl was also observed for each transporter with a replacement of Asp204. A closer examination of $K_{\text{Glc}}^{\text{app}}$ unveiled two classes of mutations. First, Glu in place of Asp204 reduced the apparent affinity for Glc in either NaCl or HCl about 4-fold. The second class encompasses transporters with a neutral side chain at position 204 (Asp204 $\rightarrow$ Asn or Cys), which showed increased apparent affinities for Glc. Although the $K_{\text{Glc}}^{\text{app}}$ of D204C in HCl was only slightly decreased (3 mM), a $\sim$5-fold reduction of $K_{\text{Glc}}^{\text{app}}$ was observed in NaCl (0.07 mM). D204N exhibited a more dramatic increase of the apparent affinity for Glc with a 10- and 15-fold reduction of $K_{\text{Glc}}^{\text{app}}$ in HCl (0.6 mM) and in NaCl (0.02 mM), respectively. Over a range from $-150$ to $-30$ mV, the $K_{\text{Glc}}^{\text{app}}$ determined in NaCl was essentially voltage-insensitive for all transporters. From $-150$ to $-50$ mV, WT exhibited an approximate 4-fold increase of $K_{\text{Glc}}^{\text{app}}$ in HCl, but no voltage dependence of the $K_{\text{Glc}}^{\text{app}}$ was observed for the other transporters.

The $f_{\text{max}}^{\text{app}}$ values for each transporter increased (more negative) with hyperpolarizing potentials and were comparable to the currents generated by 100 mM D-glucose (see Fig. 2). The data could not determine the magnitude of $f_{\text{max}}^{\text{app}}$ for WT (Table I). WT and D204E exhibited a sigmoidal $f_{\text{max}}^{\text{app}}$-$V_m$ relation in NaCl and thus, the similar $f_{\text{max}}^{\text{app}}$ values of these two transporters shown at $-110$ mV (Table I) were not significantly changed by more hyperpolarizing potentials. With the exception of WT, no saturation of the $f_{\text{max}}^{\text{app}}$-$V_m$ relationship was observed for each transporter at hyperpolarizing potentials in HCl.

To elucidate whether the substitution of Asp204 in hSGLT1 changed the sugar specificity of the transporter, oocytes were held at $-50$ mV and currents generated by 10 mM sugar were monitored in the presence of NaCl or HCl. Under either test condition the selectivity pattern for each transporter was in the order Glc $\approx$ OMDG $\approx$ D-galactose $> 3$-O-methyl-D-glucose (data not shown). Consistent with the effect of the cation on the apparent Glc affinity for WT and D204E, each sugar induced $\sim$10-fold lower currents in the presence of HCl than in NaCl.
effect on the apparent Glc affinity of D204E was reflected by ~4-fold lower sugar-induced currents in NaD and Hc than with WT. In Hc, D204C and D204N exhibited sugar-induced currents similar to WT, but the currents generated by each sugar in NaD were ~50-fold lower than the sugar-evoked currents observed for WT.

We determined the apparent half-maximal cation concentration for Na+- and H+-activated glucose transport (K_{0.5}^{cat}). Table I summarizes K_{0.5}^{cat} for all transporters at V_m = −110 mV. For WT the K_{0.5}^{cat} (1 μM, pH 5.2) was about three orders of magnitude smaller than the K_{0.5} (6 mM). Again, the nature of the substitution of Asp204 grouped transporters with a neutral amino acid side chain (Asp204 → Asn or Cys) in one group. These transporters exhibited a 10-fold reduced K_{0.5}^{cat}, but the K_{0.5} was not significantly altered. On the other hand, Glu in place of Asp204 reduced K_{0.5}^{cat} by about 3-fold (2 mM). This conservative substitution reduced K_{0.5}^{cat} of this transporter by a factor of >20, shifting the pH of the apparent half-maximal concentration for H+-activated glucose transport from pH 5.2 to ~6.5. In general, for each transporter K_{0.5}^{cat} was slightly voltage-dependent and increased about 4-fold from −150 to −50 mV, while K_{0.5}^{cat} over the same voltage range increased by a factor of about 2.

The Hill coefficient (n) of WT for cation-activated glucose transport was larger than unity for both Na+ (n_{Na} > 1.5) and H+ (n_{H} > 1.2) and was not affected by voltage (−50 to −150 mV). n_{Na} of D204E was >1.3 but for H+ /sugar cotransport n_{H} was <1. Independent on the cation, D204C and D204N exhibited a n_{Na} or n_{H} of <1.

Whereas at −110 mV V_{Na}^{max} for each transporter was essentially indistinguishable from V_{Glc}^{max} in Na+, this similarity in Hc was only observed for WT and D204E (note that the V_{Glc}^{max} for the H+ /V relation of D204C and D204N and the H+ /V relation of D204E did not saturate at hyperpolarizing potentials). On the other hand, at V_m = −110 mV, a transporter with a neutral amino acid side chain at position 204 exhibited a ~3-fold lower V_{Na}^{max} (D204C, −1630 nA; D204N, −2450 nA) than V_{Glc} in Hc (D204C, −675 nA; D204N, −769 nA) and V_{H}^{max} increased supralinearly with more negative potentials.

To determine the net Na+ and H+ leaks through each transporter, [Na+] or [H+] was varied between 0.1 and 100 mM Na+ or between 0.03 and 32 μM H+ and phlorizin-sensitive currents and choline currents at pH 8.0 were subtracted from the total currents. The kinetics of the cation leak were calculated at V_m = −110 mV by fitting the data to Eq. 2. In general, for each transporter K_{Na}^{cat} for glucose cotransport and leak were essentially identical, but the K_{0.5} value for the leak was ~10-fold smaller than K_{Na}^{cat}. WT exhibited a leak I_{Na} of ~97 ± 4 nA and replacement of Asp204 with Asn (54 ± 5 nA), Cys (18 ± 2 nA), or Glu (47 ± 3 nA) reduced the leak I_{Na} by ~2- to 5-fold. The leak I_{H}^{max} for WT (430 ± 4 nA) was 2.5 to 3 times larger than for D204C (−172 ± 16 nA) and D204N (−142 ± 7 nA), but there was apparently no H+ leak through D204E. Although the Hill coefficient (n) for the Na+ or the H+ leak pathways was ~1 only for WT, the value of n for each modified transporter was ≤1.

Pre-Steady-State Charge Movement—Fig. 3A shows representative current records of oocytes injected with WT or D204E cRNA in the presence of NaD or Hc after stepping the membrane potential from the holding potential (~50 mV) to the test potential (V_m = −150 to +50 mV in 20-mV decrements). After the initial fast membrane capacitive transients (τ < 1 ms) each transporter exhibited currents that relaxed to a steady-state with a single time constant (see Fig. 4). These relaxations were abolished after addition of saturating [sugar] and/or [phlorizin] (data not shown). The removal of a negative amino acid side chain at position 204 caused a dramatic reduction of the current transients (compare Q_{max} in Table I).

The charge movement was obtained by integration of the transporter-mediated relaxation. At each V_m, a plot of the charge (Q) versus the membrane voltage (V_m) yielded a sigmoidal charge-voltage (Q-V) curve (Fig. 3B). Data were fitted to Eq. 3 to obtain the Boltzmann parameters Q_{max} (maximal charge), V_{0.5} (V_m at which 50% of the charge has moved in the membrane electric field), and z (the apparent valence of the charge).
Q_{max} of WT and D204E were comparable in Na_c and H_2O (−23 nanocoulombs (nC)), and Q_{max} of D204C and D204N were reduced about 5-fold (Table I). The substitution of Na_c by H_c shifted V_{0.5} of WT from −36±1 mV to −74±4 mV. Glu in place of Asp^{204} exhibited a V_{0.5} in Na_c of −50±2 mV. However, the V_{0.5} of this transporter in H_2O was shifted to more positive potentials (V_{0.5} −+40±5 mV).

In Fig. 3B, the effect of varying [cation] on the charge-voltage (Q-V) relationship for WT and D204E, At [Na^+] or [H^+] below 25 mM or 3.2 μM, respectively, the Q-V curve for WT didn’t become saturated at negative potentials. D204E exhibited a sigmoidal Q-V curve over the entire [Na^+] range tested. At [H^+] higher than 3.2 μM, the Q-V relationship of the latter transporter did not become saturated at +50 mV.

Fig. 3C summarizes V_{0.5} as a function of [cation]. Reducing [Na^+] from 100 to 25 mM shifted V_{0.5} of WT from −36±1 mV to −91±2 mV. Plotting V_{0.5} as a function of log[Na^+] revealed a linear relation with a slope of 98±3 mV per 10-fold change in [Na^+] (ΔV_{0.5}^{Na}). V_{0.5} for D204E was apparently not dependent on [Na^+] (ΔV_{0.5}^{Na}) of 8.7±0.2 mV). In H^+ WT ΔV_{0.5}^{Na} of 92±7 mV), and D204E (ΔV_{0.5}^{Na} = 87±11 mV) exhibited a similar slope of ΔV_{0.5}^{Na}. However, V_{0.5} of D204E was shifted by about 110 mV toward positive potentials. Over the concentration range shown in Fig. 3C, WT and D204E exhibited a z value of −1.

Relaxation Time Constants—Subtraction of capacitive and steady-state currents from the total currents (see Fig. 3A) revealed a monoeponential time constant (τ) of the pre-steady-state relaxation currents. τ was voltage-dependent in the ON response but not dependent on voltage in the OFF response (Fig. 4). In Na_c the shapes of the τ_{ON}-V curves for the transporters tested were bell-shaped and described a Gaussian fit. The maximum (τ_{max}) for WT and D204E was −20 ms and −27 ms at −100 mV. In contrast, in H_2O the τ-V curve of WT didn’t reach τ_{max} even after the applied V_m (+50 mV to −150 mV) but the bell-shaped τ-V relation of D204E was dramatically shifted to the right (τ_{max} of −32 ms at −22 mV). As reported above, D204C and D204N exhibited very small pre-steady-state currents that were at the border of resolution and precluded us from determining reliable τ_{max} values over the entire voltage range.

Stoichiometry—The stoichiometry of ion/sugar cotransport was determined by simultaneously measuring the unidirectional flux of D-[14C]glucose into oocytes expressing WT or D204N and monitoring the sugar-evoked inward current. Integration of the inward current with time revealed the net positive charges that entered the oocyte. A plot of the net charge against glucose uptake by oocytes expressing WT revealed a linear relation in both Na_c and H_2O with a slope of −2 inward charges per glucose molecule transported (2.1±0.1 in Na_c; 2±0.3 in H_2O) (Fig. 5). In the presence of Na_c, the slope of the inward charge/glucose relation of D204N was 2.1±0.4. Replacing Na_c with H_2O changed the slope for D204N to 39±3. By reducing the final D-[14C]glucose concentration in H_2O from 5 to 0.1 mM, the slope for D204N was 144±7 (Fig. 5, inset), but no significant effect on the H^+-glucose stoichiometry was observed for WT (data not shown). No Na_c- or H_2O-dependent glucose uptake or sugar-evoked current was observed in control (H_2O-injected) oocytes.

Outward Currents—We next studied the effect of intracellular acidification on glucose-induced H^+ currents. Acidification of the intracellular compartment was produced by rapid replacement of external choline chloride with potassium acetate (21). Fig. 6 shows representative I-V curves on the effect of internal acidification of oocytes expressing D204N or WT in the presence of 31.6 μM H^+ (pH 4.5). In the absence of acetate, the glucose-induced currents for D204N increased with hyperpolarization and reversed at +23 mV (Fig. 6A). Internal acidification increased the magnitude of outward currents with depolarization (+800 nA at +50 mV) but had only minor effects on currents at potentials more negative than −70 mV. No outward currents were observed after internal acidification of oocytes expressing WT under either condition (Fig. 6B), despite
the fact that the amount of WT in the plasma membrane was about four times that of D204N (Q\text{max} for WT = 14 nC, for D204N = 4 nC). Addition of 0.2 mM phlorizin reversibly blocked the inward and outward glucose-induced currents for D204N completely (Fig. 6C).

**DISCUSSION**

The present study confirms and extends our previous reports on the kinetics of SGLT1 (4, 5): First, the steady-state and pre-steady-state kinetics of the transporter in Na\(^{+}\) are in close agreement with previous observations on hSGLT1 (10, 17, 20, 22–24). Second, we confirm and extend our findings on rabbit SGLT1 (25, 26) that H\(^{+}\) can drive sugar cotransport through hSGLT1. The kinetics of H\(^{+}\) and Na\(^{+}\) sugar cotransport are quite similar, even though the apparent affinity of hSGLT1 for H\(^{+}\) is ~1000-fold greater than for Na\(^{+}\), and the apparent affinity for sugar is about 10-fold lower in H\(^{+}\) than in Na\(^{+}\) (see Table I). In fact, the maximum rates of cotransport, the cation-to-sugar stoichiometry (2:1), the Hill coefficients (>1), turnover numbers (~50 s\(^{-1}\)), and voltage dependence of transport are virtually identical for Na\(^{+}\) and H\(^{+}\) cotransport when measured in the same oocyte. Furthermore, the pre-steady-state kinetic parameters Q\text{max}, V\text{max}, and \(\tau\) are comparable in Na\(^{+}\) and H\(^{+}\). The only quantitative differences are that the turnover number for the leak pathway (uniport) (27) is about 5-fold higher in H\(^{+}\) (22 s\(^{-1}\)) than in Na\(^{+}\) (4.4 s\(^{-1}\)) and that \(k_{\text{on}}^{{\text{leak}}}\) is about one order of magnitude smaller for the leak than for the cotransport. The latter observation is consistent with the results for hSGLT1 reported by Chen et al. (24). In contrast to their work (they reported a Hill coefficient for Na\(^{+}\) uniport = 1 and for H\(^{+}\) uniport > 1), the present study clearly emphasizes that the Hill coefficients for both Na\(^{+}\) and H\(^{+}\) uniport are >1. Together with the concentration dependence of V\(_{0.5}\) (95 mV per 10-fold change in [Na\(^{+}\)] or [H\(^{+}\)]), this finding supports our hypothesis that two cations, Na\(^{+}\) or H\(^{+}\), bind to SGLT1 before sugar. This is in direct contradiction to the Na\(^{+}\) (or H\(^{+}\))-sugar-Na\(^{+}\) binding sequence model proposed by Chen et al. (24) based on their experiments. However, Chen’s model is inconsistent with their reported results, because it does not take into account that the Hill coefficient for the H\(^{+}\) leak current was >1 and that the currents for the Na\(^{+}\) leak were no greater than 15 nA (com-
pared with 100 nA in the present study). Furthermore, their conclusion that Na⁺ is the last substrate bound to the transporter cannot account for the steady-state kinetics, because the maximum rate of Na⁺/glucose cotransport is independent of [Na⁺]

To explore the role of the N terminus of hSGLT1 in cation/sugar cotransport, we have analyzed the effect of replacing a conserved amino acid residue, Asp204. This residue is aligned with Asp187 in the Na⁺/proline cotransporter (PutP), which is functionally important for cation selectivity and Na⁺-dependent proline binding and transport (12). Replacing Asp204 revealed two functional classes of mutations, depending on the presence or absence of a negative-charged amino acid side chain at position 204. This stands in contrast to PutP, where a polar rather than a charged residue at position 187 is essential for function.

The first class of mutations consists only of a transporter with a Glu in place of Asp204. Independent of the coupling cation, the apparent affinity for glucose is increased by -4-fold, indicating a cation species-independent effect on the glucose translocation through D204E. The apparent affinity of the transporter for Na⁺ is increased by 3-fold, whereas the I-V relation and f(V)max in Na⁺, f(V)max and the leak f(V)max are all comparable to WT. These minor kinetic differences are reflected by similar turnover numbers by these transporters. On the other hand, D204E exhibits an increased apparent H⁺ affinity. This implies that lengthening the side chain at position 204 by only one methylene group (about 1.5 Å) dramatically influences, directly or indirectly, the geometry of the cation site(s). A similar observation was reported for the glutamate transporter (GLT-1 or EAAT-2) (28). The increased apparent H⁺ affinity of D204E is mirrored by the alkaline shift in the Q-V relation (Fig. 3B), suggesting that, even at very low [H⁺], binding of H⁺ induces conformational changes that are the basis for coupled transport (10). This fact is most likely the reason for the apparent independence of the Q-V relation of D204E on [Na⁺], because H⁺ is the preferred cation species. At [H⁺] larger than 3 μM no saturation at depolarizing potentials is detectable, precluding reliable calculations in this [H⁺] range. However, between 0.1 and 3 μM H⁺ ΔV10 of D204E is similar to ΔV10 for WT from 1 μM to 32 μM H⁺. The -20-fold increase in apparent affinity is also reflected by a -110 mV shift of V0.5 toward positive potentials and a τmax of D204E at -25 mV (no τmax is detectable for WT in H⁺ from -150 mV to +50 mV). According to computer simulations of the 6-state kinetic model for SGLT1 (5), this right shift of τmax and the generally higher τ values are due to higher binding and lower dissociation rate constants for the cation. This prediction is consistent with the observation that no H⁺ leak through D204E is observed and may explain why the glucose-evoked H⁺ currents (Fig. 2) were about -5-fold smaller for D204E than for WT. Because the I-V relation of D204E in H⁺ does not saturate at hyperpolarizing potentials, the turnover number for H⁺/glucose cotransport for D204E at -110 mV must be greater than 9 s⁻¹.

The second class of mutations encompasses transporters with a neutral side chain at position 204 (Asp → Asn or Cys). By using thiol labeling, the predicted salt-bridge location of Asp204 (1, 29) is confirmed, because Cys204 is only accessible from the cell inside (data not shown). Furthermore, introduction of a positive charge (by 2-aminoethyl methanethiosulfonate) doesn’t have a significant effect on the kinetics of the transporter. Based on this observation, we may exclude a possible role of Asp204 in salt-bridge formation with a positive amino acid as proposed for Asp residues in the lactose permease (LacY) (30–32). However, as indicated by the 5-fold reduction of Qmax for D204C and D204N, removal of the negative charge at position 204 reduces the number of transporters in the plasma membrane. These data imply a trafficking defect of the protein to the plasma membrane: a common problem observed with the expression of a membrane protein in an eukaryotic expression system (33, 34). This trafficking problem precludes us from a detailed analysis of the pre-steady-state kinetics of D204C or D204N.

Analysis of steady-state kinetics of D204C and D204N shows a >10-fold reduction of Kc(Glc) 0.5 in Na⁺ and H⁺. The finding that this single mutation also produces a 10-fold reduction in Kc(Na⁺) raises the idea that cation and sugar sites are in close proximity or may overlap. A similar hypothesis is proposed for the melibiose permease of E. coli, where an N-terminal domain of the transporter plays a fundamental role in connecting cation- and sugar-binding sites in terms of coupling (35, 36). Although the apparent affinity parameters of the (co)substrates for D204C and D204N were altered and the steady-state activation of the currents revealed a Hill coefficient < 1, neither the coupling ratio of Na⁺ to glucose transport (Fig. 5) nor the turnover number for Na⁺/glucose cotransport (f(V)max/Qmax) were significantly altered.

Unexpectedly, all experimental evidence indicates that neutralization of Asp204 (D204C and D204N) in hSGLT1 results in the activation of a glucose-activated H⁺ channel. This conclusion is based on the observations that: 1) The glucose-evoked H⁺ currents are an order of magnitude greater than the Na⁺ currents (Fig. 2). Because the H⁺ currents do not become saturated within the applied voltage range, this would imply that the turnover number (UH/Qmax) increases by more than one order of magnitude over that in Na⁺ (Table I). 2) The H⁺ currents are enhanced at positive voltages, unlike either the Na⁺ currents for D204C or D204N or the H⁺ and Na⁺ currents for WT or D204E (Fig. 2). These outward H⁺ currents are enhanced by internal acidification of the oocyte (Fig. 6A) and are blocked by the addition of phlorizin (Fig. 6C). 3) The H⁺ currents greatly exceed those expected for the strict coupling of H⁺/sugar cotransport. The H⁺/sugar stoichiometry increases from the expected value of 2 to as high as 144 (Fig. 5). The Na⁺/glucose stoichiometry for both WT and D204N was 2:1. 4) There is no change in the H⁺ leak currents for these mutants. The turnover numbers for the H⁺ leak currents for D204C and D204N (−172 nA/V nC = 34.5 s⁻¹; −142 nA/4.4 nC = 32 s⁻¹) are comparable to that for the wild-type protein (22 s⁻¹).

These results imply that minor structural changes in hSGLT1, i.e. replacing a carboxyl side chain with an amine or sulfhydryl group, are sufficient to open a glucose-activated H⁺ channel. This channel activity is intimately associated with sugar transport, because the competitive blocker phlorizin is unable to activate the H⁺ channel but is only able to block the effect of glucose. Clearly, based on our results we cannot distinguish whether neutralization of this conserved acidic residue generates a new “artificial” H⁺ pore or affects interactions with the coupling cation and/or other parts of the protein, thereby modifying the “original” H⁺ pathway. Because Asp204 is located in a cytoplasmic loop, it seems unlikely that this residue is part of the membrane-spanning H⁺ pathway. However, with the lack of high resolution structural data of this transporter, this question will remain an enigma.

Our observations lend support to the suggestion that cotransporters and channels share features in common. For example, the glutamate co-transporters also behave as ligand-induced chloride channels (37, 38, see also Ref. 39 for review). However, this report shows that hSGLT1 appears to be unique in that: 1) ion channel activity is only observed in the mutagenized protein; 2) the ligand (sugar) opens a channel for the driving cation (H⁺) for cotrans-
port, and 3) SGLT1 functions as a monomer (40). The glutamate cotransporter EAAT3 occurs as a homopentamer in oocyte plasma membranes, and it has been suggested that the chloride channel is formed by the oligomer (41).

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**REFERENCES**

1. Turk, E., and Wright, E. M. (1997) *J. Membr. Biol.* **159**, 1–20
2. Reizer, J., Reizer, A., and Saier, M. H., Jr. (1994) *Biochem. Biophys. Acta* **1197**, 133–166
3. Hediger, M. A., Coady, M. J., Ikeda, T. S., and Wright, E. M. (1987) *Nature* **330**, 379–381
4. Parent, L., Supplisson, S., Loo, D. D. F., and Wright, E. M. (1992) *J. Membr. Biol.* **135**, 49–62
5. Parent, L., Supplisson, S., Loo, D. D. F., and Wright, E. M. (1992) *J. Membr. Biol.* **125**, 63–79
6. Wright, E. M., Loo, D. D. F., Panayotova-Heiermann, M., Lostao, M. P., Hirayama, B. A., Mackenzie, B., Boorer, K., and Zampighi, G. (1994) *J. Exp. Biol.* **186**, 197–212
7. Wright, E. M., Loo, D. D. F., Turk, E., and Hirayama, B. A. (1996) *Curr. Opin. Cell Biol.* **8**, 468–473
8. Panayotova-Heiermann, M., Loo, D. D. F., Kong, C. T., Lever, J. E., and Wright, E. M. (1996) *J. Biol. Chem.* **271**, 19029–19034
9. Panayotova-Heiermann, M., Eskandari, S., Turk, E., Zampighi, G. A., and Wright, E. M. (1997) *J. Biol. Chem.* **272**, 20324–20327
10. Loo, D. D. F., Hirayama, B. A., Gallardo, E. M., Lam, J., Turk, E., and Wright, E. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7789–7794
11. Quick, M., and Jung, H. (1997) *Biochemistry* **36**, 4631–4636
12. Quick, M., and Jung, H. (1998) *Biochemistry* **37**, 13800–13806
13. Quick, M., Stoeßling, S., and Jung, H. (1999) *Biochemistry* **38**, 13523–13529
14. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1994) *Anal. Biochem.* **215**, 363–370
15. Hattori, M., and Sakaki, Y. (1986) *Anal. Biochem.* **152**, 223–238
16. Ikeda, T. S., Hwang, E. S., Coady, M. J., Hirayama, B., Hediger, M. A., and Wright, E. M. (1989) *J. Membr. Biol.* **110**, 87–95
17. Loo, D. D. F., Hazama, A., Supplisson, S., Turk, E., and Wright, E. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5767–5771
18. Mackenzie, B., Loo, D. D. F., and Wright, E. M. (1998) *J. Membr. Biol.* **162**, 101–106
19. Hazama, A., Loo, D. D. F., and Wright, E. M. (1997) *J. Membr. Biol.* **155**, 175–186
20. Hirayama, B. A., Loo, D. D. F., Panayotova-Heiermann, M., Loo, D. D. F., Turk, E., and Wright, E. M. (1996) *Am. J. Physiol.* **270**, G919–G926
21. Zampighi, G. A., Loo, D. D. F., Kerman, M., Eskandari, S., and Wright, E. M. (1999) *J. Gen. Physiol.* **113**, 507–523
22. Chen, X.-Z., Coady, M. J., Jackson, F., Berteloot, A., and Lapointe, J.-Y. (1995) *Biophys. J.* **69**, 2405–2414
23. Chen, X.-Z., Coady, M. J., and Lapointe, J.-Y. (1996) *Biophys. J.* **71**, 2544–2552
24. Chen, X.-Z., Coady, M. J., Jalal, F., Wallendorf, B., and Lapointe, J.-Y. (1997) *Biophys. J.* **73**, 2505–2510
25. Hirayama, B. A., Loo, D. D. F., and Wright, E. M. (1994) *J. Biol. Chem.* **269**, 21407–21410
26. Hirayama, B. A., Loo, D. D. F., and Wright, E. M. (1997) *J. Biol. Chem.* **272**, 2110–2115
27. Loo, D. D. F., Hirayama, B. A., Meinild, A.-K., Chandy, G., Zeuthen, T., and Wright, E. M. (1999) *J. Physiol.* **518**, 195–202
28. Kavanagh, M. P., Bendahan, A., Zerangue, N., Zhang, Y., and Kanner, B. I. (1997) *J. Biol. Chem.* **272**, 1703–1708
29. Turk, E., Kerner, C. J., Loo, D. M. P., and Wright, E. M. (1996) *J. Biol. Chem.* **271**, 1925–1934
30. Dunten, R. L., Sahin-Töth, M., and Kaback, H. R. (1993) *Biochemistry* **32**, 3139–3145
31. Sahin-Töth, M., and Kaback, H. R. (1993) *Biochemistry* **32**, 10027–10035
32. Lee, J. L., Hwang, P. P., Hansen, C., and Wilson, T. H. (1992) *J. Biol. Chem.* **267**, 20753–20764
33. Lam, J. T., Martin, M. G., Turk, E., Hirayama, B. A., Bosshard, N. U., Steinmann, B., and Wright, E. M. (1999) *Biochem. Biophys. Acta* **1453**, 297–303
34. Lostao, M. P., Hirayama, B. A., Panayotova-Heiermann, M., Sampogna, S. L., Bok, D., and Wright, E. M. (1995) *FEBS Lett.* **377**, 181–184
35. Ambròise, Y., Leblanc, G., and Rousseau, B. (2000) *Biochemistry* **39**, 1338–1345
36. Cordat, E., Leblanc, G., and Mus-Veteau, I. (2000) *Biochemistry* **39**, 4493–4499
37. Wadiche, J. I., Amara, S. G., and Kavanaugh, M. P. (1995) *Neuron* **15**, 721–728
38. Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) *Nature* **375**, 599–603
39. Sonders, M. S., and Amara, S. G. (1996) *Curr. Opin. Neurobiol.* **6**, 294–302
40. Eskandari, S., Wright, E. M., Kerman, M., Starace, D. M., and Zampighi, G. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11235–11240
41. Eskandari, S., Kerman, M., Kavanaugh, M. P., Wright, E. M., and Zampighi, G. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8641–8646
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