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Identification of a Region Critically Involved in the Interaction of Phlorizin with the Rabbit Sodium-D-Glucose Cotransporter SGLT1

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Abstract. In order to define potential interaction sites of SGLT1 with the transport inhibitor phlorizin, mutagenesis studies were performed in a hydrophobic region of loop 13 (aa 604–610), located extracellularly, close to the C-terminus. COS 7 cells were transiently transfected with the mutants and the kinetic parameters of α-methyl-D-glucopyranoside (AMG) uptake into the cells were investigated. Replacement of the respective amino acids with lysine reduced the maximal uptake rate: Y604K showed 2.2%, L606K 48.4%, F607K 15.1%, C608K 13.1%, G609K 14.1%, and L610K 17.2% of control. In all mutants the apparent K_i for phlorizin increased at least by a factor of 5 compared to the wild-type K_i of 4.6 ± 0.7 μmol/l; most striking changes were observed for Y604K (K_i = 75.3 ± 19.0 μmol/l) and C608K (K_i = 83.6 ± 13.9 μmol/l). Replacement of these amino acids with a non-polar amino acid instead of lysine such as in Y604F, Y604G and C608A showed markedly higher affinities for phlorizin. In cells expressing the mutants the apparent affinity of AMG uptake for the sugar was not statistically different from that of the wild type (K_m = 0.8 ± 0.2 mmol/l).

These studies suggest that the region between amino acids 604 and 610 is involved in the interaction between SGLT1 and phlorizin, probably by providing a hydrophobic pocket for one of the aromatic rings of the aglucone moiety of the glycoside.

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Introduction

Phlorizin, a glycoside present in the bark of fruit trees, is a well known inhibitor of renal D-glucose transport. Since the first observations of Lotspeich (1961), Diedrich has extended the studies employing various analogues (1963, 1972) and recently in our laboratory a pharmacophore model of phlorizin has been developed (Wielert-Badt et al., 2000). In various transport and binding studies it has been shown that phlorizin inhibits sodium-D-glucose cotransport in a competitive manner (Bode, Baumann, Diedrich, 1972; Diedrich, 1963; Frasch et al. 1970; Lin & Hahn, 1983; Vick et al. 1973) and it is postulated that both, the sugar binding site of the transporter and a binding site for the aglucone moiety, are involved in the interaction of the carrier with the inhibitor (Wielert-Badt et al., 2000). Thereby, hydrogen bonds (mainly for the sugar moiety) as well as hydrophobic interactions (mainly for the aglucone moiety) have been invoked.

It also has been reported that cysteines play a major role in this process since N-ethylmaleimide (NEM) has been shown to inhibit phlorizin binding and that NEM-reactive sites can be protected by the presence of phlorizin (Kinne, 1976; Thomas, Kinne, Frohnert, 1971; Turner & George, 1983; Turner & George, 1984). Recent studies from our laboratory employing site-directed mutagenesis support this notion (unpublished observations). The regions of the transporter protein that are involved in this interaction have, however, not yet been unequivocally identified.

The studies of Panayotova-Heiermann et al. (1996, 1997) and Wright (2001) suggest that the D-glucose translocation takes place in the C-terminal part of the

protein and probably involves the transmembrane helices 10–13. Thus, the peptide loops connecting these helices are most likely involved in the binding of phlorizin to the carrier. Since recent studies on the topology of the transporter (Lin et al., 1999), immunoprecipitation (Lin et al., 1998), the reactivity of cysteines to MTSET, and molecular recognition force microscopy (unpublished observations) suggest an extracellular orientation of loop 13, we concentrated in the studies presented below on hydrophobic amino acids located in this loop. By site-directed mutagenesis the character of the amino-acid side chains was altered and the effect of these replacements on the kinetic constants of AMG-uptake into transiently transfected COS 7 cells was investigated. The results obtained suggest that the region between aa 604 and 610 in loop 13 plays a significant role in the interaction of phlorizin with the transporter.

Materials and Methods

Mutagenesis of SGLT1 cDNA

All mutants were prepared by site-directed mutagenesis using Chameleon™ Double-Stranded Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The following primers with mutated nucleotides (underlined) were used for mutagenesis:

D553A, 5′-CTTCACCAAGCCCATTCCA**GCT**GTGCATCTCTATCGTCTGTGC-3′

D572A, 5′-GCAAAGAGGAGCGCATT**GCC**TTGGATGCAGGAGAGG-3′

Y604G, 5′-GGATTCTTCAGGCGGGCC**GGA**GACCTGTTTTGTGGGCTG-3′

Y604F, 5′-TTCTTCAGGCGGGCC**TTC**GACCTGTTTTGTGGGCTG-3′

Y604K, 5′-GGATTCTTCAGGCGGGCC**AAG**GACCTGTTTTGTGGGCTG-3′

L606K, 5′-CAGGCGGGCCTATGAC**AAG**TTTTGTGGGCTGGAC-3′

F607K, 5′-CGGGCCTATGACCTG**AAG**TGTGGGCTGGACCAG-3′

C608K, 5′-GCCTATGACCTGTTT**AAG**GGGCTGGACCAGGATAAG-3′

G609K, 5′-GCCTATGACCTGTTTTGT**AAG**CTGGACCAGGATAAG-3′

L610K, 5′-GACCTGTTTTGTGGG**AAG**GACCAGGATAAGGGACCCAAG-3′

Plasmid Construction

For preparation of mutants, plasmid pSGLT1 (2207 bp rabbit-DNA) was used. After mutagenesis all mutants were confirmed by DNA sequencing and cloned from plasmid pSGLT1 digested with *Hind*III and *Xba*I restriction enzymes, and ligated into expression plasmid pHOOK™-2 (Invitrogene, NV Leek, Holland) digested with the same enzymes. Final plasmids were purified through Qiagen endotoxin-free Plasmid Maxi Kit (Qiagen, Hilden, Germany).

Cell Culture and Transient Transfection

COS 7 cells were subcultured in six-well culture plates to 80% confluency. Transient transfection was performed by using the activated-dendrimer transfection reagent SuperFect™ (Qiagen). Post-transfection time was 48 hours. The efficiency of transfection was tested in COS 7 cells that were simultaneously transfected with the pHook™-2*lac*Z plasmid expressing β-galactosidase under identical conditions (Invitrogen, Carlsbad, CA). The transiently transfected COS 7 cells were stained with the β-galactosidase staining kit (Invitrogen) according to the manufacturer's manual. The transfection efficiency was calculated by obtaining the ratio between the number of blue cells and the total number of cells. A transfection efficiency of 35–40% was found for the wild type and all mutants.

α-Methyl-D-Glucopyranoside Uptake

Prior to the transport assays COS 7 cells were incubated in a D-glucose-free medium for 2 hours at 37°C to remove the extracellular D-glucose and to reduce the intracellular glucose concentration. α-Methyl-D-glucopyranoside uptake was measured at 37°C in KRH (Krebs-Ringer-HEPES)-Na medium or KRH-N-methylglucamine medium at a concentration of 0.1 mmol/l AMG in the absence and presence of 0.5 mmol/l phlorizin (Lin et al., 1998). The solutions contained 2 μCi/ml of the isotope. For mutants exhibiting low uptake rates the amount of isotope was increased to 5μCi/ml. The KRH-Na medium contained in mmol/l: 120 NaCl, 4.7 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$ and 10 HEPES, the pH was adjusted to 7.4 with Tris-base. In the N-methylglucamine medium NaCl was replaced by N-methylglucamine and the pH was adjusted with HCl. The transport was stopped after incubation for 15 min at 37°C by removing the medium and rinsing with ice-cold KRH-Na buffer containing 0.5 mmol/l phlorizin.

In kinetic measurements for the determination of the apparent K_m two substrate concentrations, in most cases 0.05 mmol/l (S_1) and 2 mmol/l AMG (S_2) were used. Assuming that the Michaelis-Menten equation is valid under these experimental conditions for the sodium-dependent AMG-uptake, the following equation was used to calculate K_m:

$$K_m = S_1 \cdot S_2 \cdot (V_2 - V_1) / (V_1 \cdot S_2 - V_2 \cdot S_1). \quad (1)$$

To determine the apparent K_i for phlorizin, two concentrations 0.5 μmol/l (I_1) and 10 μmol/l (I_2) of phlorizin, were used at the low AMG concentration. K_i was calculated according to the equation:

$$K_i = K_m \cdot (V_{I2} \cdot I_2 - V_{I1} \cdot I_1) / (S_2 + K_m) \cdot (V_{I1} - V_{I2}), \quad (2)$$

assuming competitive inhibition. To calculate the maximal uptake rate (R_{max}) the apparent K_m values derived from the equation above were used:

$$R_{max} = (K_m + S_1) \cdot (V_1/S_1) \text{ or} \quad (3)$$

$$R_{max} = (K_m + S_2) \cdot (V_2/S_2). \quad (4)$$

Each experiment was repeated several times on different days. Mean values ± SEM are given throughout the paper.

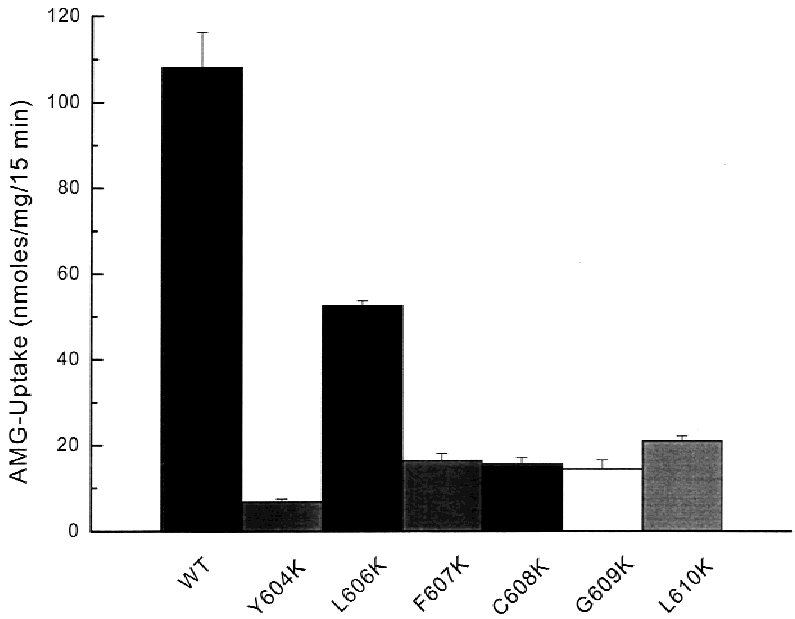

Fig. 1. Effect of the introduction of lysine in the positions 604–610 of SGLT1 on Na^+-dependent AMG-uptake into COS 7 cells transiently transfected with the indicated mutants. The AMG concentration was 0.1 mmol/l. Mean values from 4 experiments are given. (WT = wild type SGLT1).

Isolation of RNA and Northern Blot Hybridization

Total RNA from COS 7 cells was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany). Five micrograms of RNA were loaded on an 1.2% agarose gel in the presence of 16% formaldehyde. After electrophoresis RNAs were transferred on nylon membrane (Boehringer Mannheim GmbH, Germany), hybridized with the 1200 bp *Nco*1 fragment from sglt1 gene labeled with digoxigenin (Boehringer Mannheim GmbH, Germany). As control a 450 bp PCR-amplified fragment from the D-glyceraldehyde-3-phosphate dehydrogenase from COS 7 cells was used for hybridization under identical conditions.

Materials

The COS 7 cell line (SV40 transformed) was purchased from the European Collection of Cell Culture Center, Salisbury, UK. ^{14}C-AMG (specific activity 261 mCi/mmol) was obtained from NEN (Boston, MA, USA). Phlorizin was obtained from Sigma (Steinheim, Germany). It was dissolved in ethanol as a stock solution at a concentration of 10 mmol/l and kept at −20°C. Other chemicals were of the highest purity commercially available.

Results

Transfection and Expression

To be sure that all constructed plasmids were successfully transcribed, mutants of loop 13 (*see* Table 1 and 2) were transiently transfected into COS 7 cells as described in Methods. Expression at the transcriptional level was examined by Northern blot analysis using 1200 bp *Nco*1-fragment of the *sglt*1 gene as a probe for hybridization. As a control a 450 bp PCR amplified fragment from the D-glyceraldehyde-3-phosphate dehydrogenase from COS 7 cells was used for hybridization under identical conditions. In these screening experiments, all plasmids induced the production of similar levels of mRNA (*data not shown*).

Hydrophobicity Analysis of Loop 13

In order to determine hydrophobic regions constituting potential phlorizin binding sites we first analyzed loop 13 with regard to the existence of clusters of aromatic amino acids. Using the Kyte and Doolittle (Kyte & Doolottle, 1982) algorithm the area with the highest hydrophobicity was found to be located between aa 604 and 610. We therefore chose these amino acids for site-directed mutagenesis studies.

Effect of Replacing Amino Acids 604 to 610 by Lysine on the Kinetic Parameters of AMG-uptake into Transiently Transfected COS 7 Cells

In Fig. 1 the uptake of AMG by COS 7 cells transfected with Y604K, L606K, F607K, C608K, G609K, or L610K is shown. All replacements decreased the cellular uptake. The most prominent effect was observed for Y604K.

In a more detailed kinetic study summarized in Table 1 the cells transfected with the mutants show also lower maximal uptake rates. Since, as described below, these reductions do not correlate with changes in the other kinetic parameters, they were not further investigated.

As depicted in Table 1 these replacements also

Table 1. Kinetic properties of AMG-uptake into COS 7 cells transiently transfected with SGLT1 mutants: Mutants from the region Y604 to L 610 in which the respective amino acid was replaced by lysine

Mutant	Apparent K_i Phlorizin (μmol/l)	Apparent K_m AMG (mmol/l)	Maximal uptake rate (nmoles/mg protein/15 min)	Number of experiments
WT	4.6 ± 0.7	0.8 ± 0.2	103.4 ± 30.1	10
Y604K	75.3 ± 19.0	1.8 ± 1.7	2.3 ± 1.7	8
L606K	26.9 ± 6.9	1.2 ± 0.1	49.9 ± 19.2	4
F607K	48.5 ± 7.6	1.6 ± 0.5	15.6 ± 2.4	6
C608K	83.6 ± 13.9	0.7 ± 0.3	13.5 ± 2.6	6
G609K	60.2 ± 4.8	3.6 ± 1.1	14.5 ± 6.1	7
L610K	30.6 ± 3.3	1.9 ± 1.2	17.7 ± 13.4	4

strongly affected the affinity of the transporter for phlorizin. The apparent K_i values for phlorizin were at least fivefold higher than the apparent K_i of the wild type. Most striking changes were observed for Y604K and C608K. Within the string of amino acids from tyrosine at 604, leucine at 606, phenylalanine at 607, cysteine at 608, glycine at 609 and leucine at 610, the apparent affinity for phlorizin shows two minima, one at Y604K and one at C608K. Then the affinity increases again. Thus, a tentative sequence for the loss of affinity can be compiled. It starts with C608K, followed by Y604K, G609K, F607K, L610K, and L606K.

Complete dose response curves for the inhibition of AMG-uptake into COS 7 cells transfected with the various mutants are shown in Fig. 2. Here, Y604K and G609K, showed no inhibition in the range of phlorizin concentrations tested. AMG uptake by F607K, C608K and L610K cells was increasingly inhibited by phlorizin. Thus, except for the cysteine mutant, the same tentative order of potency as derived from the experiments presented above is obtained, in which only two concentrations of phlorizin were used and a fully competitive nature of inhibition was assumed (*see* Methods). The experiments presented in Fig. 2 were performed at a high (5 mmol/l) AMG concentration. Under these conditions mainly the aglucone part of the phlorizin molecule contributes to the interaction with the carrier. Therefore the apparent K_i values from Table 1 and the IC_{50} values that can be derived from the dose response curves can be compared only to a limited extent.

Effect of Other Mutations on the Apparent Phlorizin Affinity of AMG-uptake into Transiently Transfected COS 7 Cells

For the two positions at which an introduction of lysine showed the strongest effect on the phlorizin sensitivity, we further investigated whether the original amino acid itself was essential for the interaction of phlorizin with the carrier or whether the introduction of lysine caused the observed shift in apparent affinity. As shown in Table 2, replacement by nonpolar amino acids as in Y604G or Y604F reconstituted the normal high affinity for phlorizin. After an exchange of C608 against alanine the transporter exhibited still a lower affinity than the wild type but the apparent affinity was strikingly higher than that of C608K. Thus, in both instances, the presence of lysine in these positions and not the loss of the original amino acids seems to induce the functional changes of the transporter.

Effect of Mutations in Amino Acids 604 to 610 on the Apparent Affinity to AMG

Interestingly, as also shown in Table 1, none of the mutations changed significantly the apparent affinity of the transport system for the sugar AMG. This result suggests that mainly the interaction of the transport protein with the aglucone part of the phlorizin molecule was changed by the point mutations. It also indicates that the sodium affinity of the transporter is not altered drastically by the manipulations since a change in sodium affinity would be expected to cause also a change in AMG affinity.

Discussion

In the studies presented above, amino acids from position 604 to position 610 were identified as a region of loop 13 where the introduction of a strongly hydrophilic lysine instead of the original amino acid with low hydrophilicity drastically reduced the apparent affinity of the SGLT1 to phlorizin but not to AMG. The introduction of the lysine residue represents both, an introduction of a bulky side chain and of a positive charge, if one assumes that the terminal amino group is protonated. With regard to the size of the amino-acid side chain, tyrosine at 604, leucine at 606 and 610 and phenylalanine at 607 are comparable with the lysine residue. Thus, in these instances, most probably the change from a hydrophobic to a hydrophilic moiety can be made responsible for the change in the reactivity of the transporter to phlorizin. For the tyrosine in position 604 the change in polarity is most extreme from a negatively charged hydroxyl group to a positively charged amino group. This might explain the very strong effect on the apparent phlorizin affinity. For the glycine at position 609, which also plays a major role in determining the affinity for phlorizin, the increased size of the side chain as well as the introduction of a positive charge might contribute to the observed effect. This glycine is immediately adjacent to cysteine 608, the replacement of which also reduces the phlorizin affinity. Since we have recently found that this cysteine forms a disulfide bond with cysteine 255 in loop 5 (un-

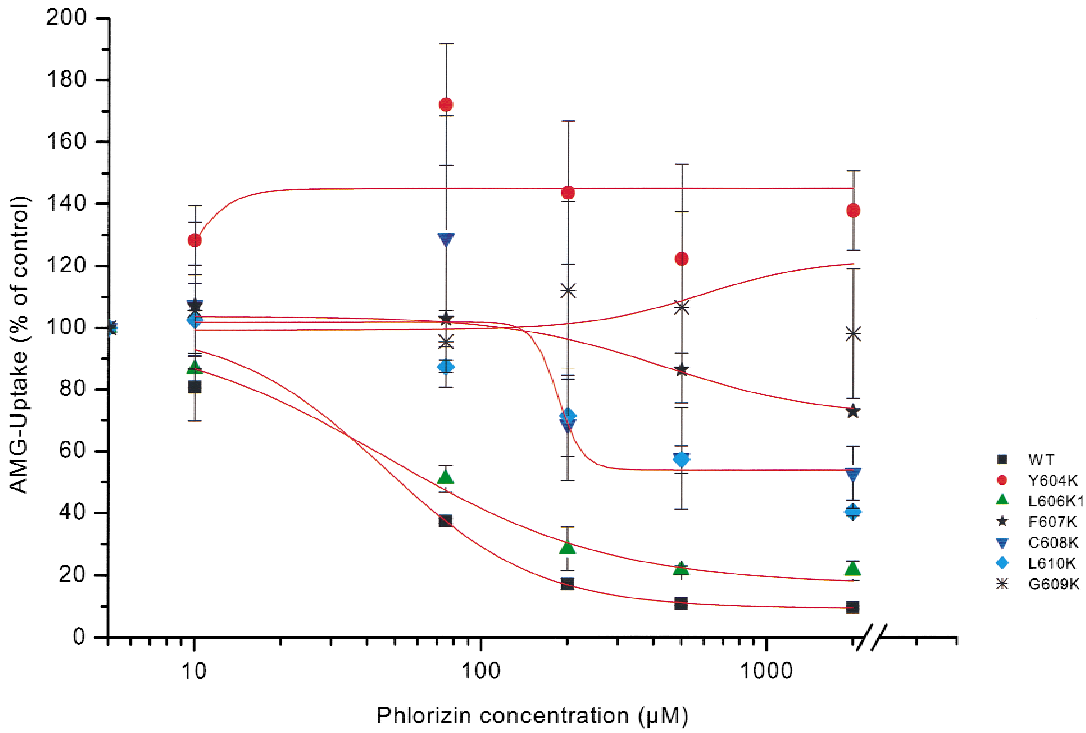

Fig. 2. Dose-response curves of phlorizin inhibition of sodium-dependent AMG-uptake into COS 7 cells transiently transfected with plasmids of various mutants of SGLT1, in which lysine had been introduced. The AMG-uptake into the cells was measured in the presence of 5 mmol/l α-methyl-D-glucopyranoside with or without 120 mM NaCl. The uptake in the presence of various phlorizin concentrations was compared to the uptake in the absence of phlorizin (set as 100%). Mean values from 4 experiments are given. (WT = wild type SGLT1).

Table 2. Kinetic properties of AMG-uptake into COS 7 cells transiently transfected with SGLT1 mutants: Mutants from the region Y604 and C608 in which the respective amino acid was replaced by nonpolar amino acids

Mutant	Apparent K_i Phlorizin (µmol/l)	Apparent K_m AMG (mmol/l)	Maximal uptake rate (nmoles/mg protein/15 min)
WT	4.6 ± 2.3	0.8 ± 0.2	103.4 ± 30.1
Y604G	3.8 ± 0.4	0.4 ± 0.1	78.7 ± 7.9
Y604F	4.4 ± 0.8	0.6 ± 0.2	120.9 ± 7.6
C608A	10.2 ± 2.5	0.7 ± 0.2	40.3 ± 5.9

Results derived from 4 experiments are shown.

published data), the lysine side chain in the position 609 might lead to pronounced changes in the arrangement of these two loops and/or the conformation of loop 13.

With regard to cysteine 608 it is noteworthy that the replacement by lysine has a larger effect on the apparent phlorizin affinity than the replacement by alanine. However, both mutants have a lower affinity for phlorizin than the wild type. This phenomenon might be explained by the assumption that the removal of the disulfide bond already interferes with phlorizin binding—as suggested by Turner and George (1983, 1984)—but that the introduction of the hydrophilic lysine group impedes the cooperation of the adjacent hydrophobic side chains in phlorizin-binding, whereas alanine, due to it's nonpolar character, does not disturb this process.

Phlorizin consists of a pyranoside ring and two aromatic rings joined by an alkyl spacer. According to previous studies [Wielert Badt et al., 2000], hydrogen bonds from the 2-, 3-, 4-, and 6-hydroxyl group of the pyranoside ring and from the 4′- and 6′-OH groups of the aromatic ring adjacent to the sugar moiety, and hydrophobic elements in the same two structures are involved in phlorizin binding. Our studies have identified one region of the SGLT1 molecule that might be involved in the hydrophobic interactions with phlorizin. Probably an interaction with one of the aromatic rings of the aglucone occurs since the AMG-affinity of the transporter was not affected by the point mutations, making a change in the D-glucose binding site unlikely. Additional studies are necessary using, for example, protein crystallography or NMR technology to identify the interacting partners of the inhibitor molecule and of the protein and to provide information on the conformation and dimensions of the interaction site.

Furthermore, the reason for the reduced maximal uptake rate of most of the cells transfected with a mutant

remains to be elucidated. Despite identical transcription of the transfected DNA, as indicated by similar mRNA levels, the rate of synthesis, the turnover of the transporter protein and/or the intracellular sorting might be affected. Those transporter molecules that reach the plasma membrane seem to be, however, functionally relatively intact, as suggested by the lack of change in their apparent affinity to AMG, although changes in the V_{max} of the transporter molecule itself cannot be excluded.

In any event, these studies provide evidence that amino acids 604 to 610 may be part of the pocket that is formed when phlorizin binds to the SGLT1 and are thereby positioned close to the D-glucose-binding and/or -translocation site. This assumption is not unlikely since the transmembrane domain 13 adjacent to loop 13 is supposed to be part of the transport pore for D-glucose (Wright, 2001).

Finally, it should be mentioned that the amino acids investigated in the current study are all conserved in the human, pig, rat and sheep SGLT1. With regard to their presence in SGLT2, which is also inhibited by phlorizin, the motif FCG is also present in all sequences known thus far. In SGLT2, this motif is flanked by methionine or tryptophane (position 606) and methionine (position 610) instead of the leucine found in SGLT1. Thus, this region might indeed be important for the binding of phlorizin to both classes of the sodium-D-glucose cotransporter, although the reason for the difference in affinity of the two types of transporters for phlorizin remains to be elucidated.

In this respect it is noteworthy that a random search for potential hydrogen bond donors also postulated to be involved in phlorizin binding (Wielert-Badt et al. 2000) thus far has been unsuccessful. Replacement of glutamic acid in positions 553 or 572 by alanine resulted in a reduced maximal uptake rate, but the apparent affinities for AMG and phlorizin remained unaltered. The respective values were: maximal uptake rate 17.3 ± 6% of control, apparent K_{AMG} 0.8 ± 0.1 mmol/l and apparent K_{iPhl} 4.0 ± 0.3 μmol/l for D553A and 14.3 ± 3%, 1.9 ± 0.7 mmol/l and 4.6 ± 2.2 μmol/l for D572A (n = 4).

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