Functional studies of the rabbit intestinal Na^+/glucose carrier (SGLT1) expressed in COS-7 cells: evaluation of the mutant A166C indicates this region is important for Na^+-activation of the carrier

Steven VAYRO, Bryan LO and Mel SILVERMAN[1]

Medical Research Council (M.R.C.) Membrane Biology Group, Department of Medicine, University of Toronto, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8 Canada

We have exploited two mutants of the rabbit intestinal Na^+/glucose carrier SGLT1 to explore the structure/function relationship of this Na^+/glucose transporter in COS-7 cells. A functional N-terminal *myc*-epitope-tagged SGLT1 protein was constructed and used to determine the plasma-membrane localization of SGLT1. The kinetic and specificity characteristics of the *myc*-tagged SGLT1 mutant were identical with those of wild-type SGLT1. Immunogold labelling and electron microscopy confirmed the topology of the N-terminal region to be extracellular. Expression of the SGLT1 A166C mutant in these cells showed diminished levels of Na^+-dependent α-methyl-D-glucopyranoside transport activity compared with wild-type SGLT1. For SGLT1 A166C, V_{max} was 0.92 ± 0.08 nmol/min per mg of protein and K_m was 0.98 ± 0.13 mM; for wild-type SGLT1, V_{max} was 1.98 ± 0.47 nmol/min per mg of protein and K_m was 0.36 ± 0.16 mM. Significantly, phlorrhizin (phloridzin) binding experiments confirmed equal expression of Na^+-dependent high-affinity phlorrhizin binding to COS-7 cells expressing SGLT1 A166C or wild-type SGLT1 (B_{max} 1.55 ± 0.18 and 1.69 ± 0.57 pmol/mg of protein respectively); K_d values were 0.46 ± 0.15 and 0.51 ± 0.11 μM for SGLT1 A166C and wild-type SGLT1 respectively. The specificity of sugar interaction was unchanged by the A166C mutation. We conclude that the replacement of an alanine residue by cysteine at position 166 has a profound effect on transporter function, resulting in a decrease in transporter turnover rate by a factor of 2. Taken as a whole the functional changes observed by SGLT1 A166C are most consistent with the mutation having caused an altered Na^+ interaction with the transporter.

INTRODUCTION

Sodium glucose co-transporters are located on the brush border membrane of intestinal and renal epithelial cells. They are responsible for secondary active uptake of sugar across this membrane. Since the cloning of the rabbit intestinal Na^+/glucose carrier (SGLT1) in 1987 [1] the transporter has been characterized in some detail (reviewed in [2–4]). Recently the topology of the human intestinal Na^+/glucose carrier has been predicted with N-glycosylation scanning mutagenesis [5]. Much of the current understanding of how the Na^+/glucose carrier functions has been obtained from electrophysiological measurements of SGLT1 expressed in *Xenopus* oocytes. On the basis of these studies a six-stage kinetic model for the operation of the transporter has been postulated [6,7].

Site-directed mutagenesis of SGLT1 has yielded a limited amount of information about its functional domains. Replacement of aspartate-176 with alanine in rabbit SGLT1 alters the kinetics of charge transfer without affecting Na^+/glucose transport kinetics, indicating that polar residues within this putative membrane-spanning domain (M4) of SGLT1 might be important in the translocation of charge [8]. A chimaeric cDNA construct that encodes residues 1–380 of the pig kidney cell line (LLC-PK_1) low-affinity Na^+/glucose transporter (SGLT2) and residues 381–662 of the LLC-PK_1 high-affinity co-transporter (SGLT1) has been functionally expressed in *Xenopus* oocytes [9]. By using the two-electrode voltage clamp method, the region identified for the sugar binding was found distal to residue 380 [9]. Confirmation that the sugar translocation pathway was indeed the C-terminal half of SGLT1 was recently demonstrated with a truncated isoform of SGLT1 (C_5), which comprised the last five transmembrane helices (helices 10–14) of the carrier, expressed in oocytes [10]. However, the region(s) of SGLT1 associated with Na^+-binding were still unknown.

In our laboratory we have initiated a systematic cysteine-scanning mutagenesis project of the rabbit intestinal Na^+/glucose carrier. The technique of cysteine-scanning mutagenesis followed by chemical modification has previously been employed to investigate the function, secondary structure and topology of cloned ion channels, or receptors [11–16]. We have obtained a series of single-cysteine-substituted SGLT1 mutants, some of which display functional characteristics distinct from those of wild-type SGLT1. For example, in a recent paper Lo and Silverman [17] provide a detailed report on the pre-steady-state electrophysiological behaviour of the A166C mutant expressed in *Xenopus* oocytes, by using the two-electrode voltage clamp method. Interestingly, this mutant displays both a decrease in substrate affinity for the transporter and a decrease in charge transfer kinetics compared with those of wild-type SGLT1. In addition, there are substantial changes in the SGLT1 A166C mutant when it is covalently modified by the thiol-specific methanethiosulphonate derivative methanethiosulphonate ethyl amine but not methanethiosulphonate ethyl sulphonate [17]. Neither of these methanethiosulphonate compounds affects wild-

Abbreviation used: α-MG, α-methyl-D-glucopyranoside.

[1] To whom correspondence should be addressed (e-mail: Melvin.Silverman@utoronto.ca).

type SGLT1, indicating that the effects of these thiol reagents can be attributed to the reactivity of the cysteine residue introduced at position 166.

These electrophysiological studies have provided us with substantial information about the sugar-induced Na^+ currents stimulated by SGLT1 A166C compared with wild-type SGLT1 and are persuasive that the single cysteine mutation at the 166 position affects Na^+ binding to the carrier. However, in this study [17] the number of functional transporters expressed at the oocyte surface could not be measured independently but had to be calculated on the basis of the assumption that all the charge transfer across the membrane was due to the carrier.

Given the potential importance of this observation, namely that the A166C mutation was affecting Na^+ interaction with the carrier, we thought it was necessary to explore in greater detail the functional characteristics of the A166C mutant compared with wild-type SGLT1. The COS-7 cell line seemed an ideal expression system because previous work [18] had shown that these cells did not possess endogenous Na^+/glucose transporters. The COS-7 cell system would allow us to complement the sugar [i.e. α-methyl-D-glucopyranoside (α-MG)]-induced Na^+-current electrophysiological data in oocytes with Na^+-dependent α-MG radioisotope measurements. Most importantly it would allow us to obtain new information about A166C including (1) an independent measure of the number of functional Na^+/glucose transporters expressed at the surface by determination of Na^+-dependent phlorrhizin (phloridzin) binding, a measurement not possible in the oocyte, and (2) an opportunity to survey for any alterations in the specificity of α-MG uptake with the sugar-binding site on SGLT1.

In the present paper we report the transient transfection of COS-7 cells with the eukaryotic expression vector pMT4, containing the SV40 promoter, encoding either wild-type SGLT1 or A166C cDNA. We also studied the distribution of SGLT1 at the COS-7 cell plasma membrane by using a novel recombinant SGLT1 protein containing a short 12-residue epitope sequence, MEQKLISEEDLV, encoding part of the human c-*myc* proto-oncogene product [19] on the N-terminal region of the protein. This is the first report of a functionally expressed N-terminal *myc*-tagged SGLT1 protein possessing identical properties with those of wild-type SGLT1. Immunogold labelling of *myc*-tagged SGLT1 and electron microscopy confirmed the topology of the N-terminal region to be extracellular and also confirmed that in COS-7 cells SGLT1 was expressed at the level of the plasma membrane.

When the SGLT1 A166C mutant was expressed in COS-7 cells it exhibited both a decrease in sugar transport affinity (K_m) and in the rate of maximal substrate transport activity (V_{max}) compared with cells expressing wild-type SGLT1. In addition, the 2:1 Na^+–glucose coupling stoichiometry and the specified sugar reactivity were the same for mutant SGLT1 and wild-type SGLT1. Significantly, there was no difference in the phlorrhizin binding affinities or the levels of maximal phlorrhizin binding between mutant and wild-type transporters.

Collectively these results suggest that (1) the A166C mutant is expressed at the cell surface to the same extent as wild-type SGLT1; (2) the A166C mutation has resulted in a significant dysfunction of SGLT1 as indicated by both a decreased sugar transport affinity and maximal rate of transport, and because the number of Na^+-dependent phlorrhizin-binding sites is unchanged this implies that the A166C mutation has resulted in a decreased carrier turnover rate; and (3) the mutation is in a region that does not affect sugar binding because both the K_d for Na^+-dependent phlorrhizin binding and the sugar specificity for wild-type SGLT1 and the A166C mutation remain unaltered. Thus we conclude that the mutation of a single cysteine residue at position 166 of SGLT1 has affected the Na^+ interaction with SGLT1 leading to a marked change in its kinetic behaviour.

MATERIALS AND METHODS

Cell culture

COS-7 cells were grown in complete RPMI 1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 21 mM $NaHCO_3$, 25 mM Hepes/NaOH, pH 7.4, 10% (v/v) foetal calf serum and 50 units/ml antibiotic solution containing penicillin and streptomycin. Cells were maintained at 37 °C in air/CO_2 (19:1). Stock cultures were grown in 75 cm^2 flasks (Corning, Cambridge, MA, U.S.A.) and were fed every 3–4 days. For transfection reactions confluent cells were trypsinized in Hanks medium (Gibco) containing 0.025% (v/v) trypsin. The cell suspension was pelleted and resuspended in 5 ml of complete RPMI medium (approx. 1.2×10^6 cells/ml). Cells were seeded into either 12-well plates (22 mm diameter; Corning) containing 1 ml of complete RPMI medium at a density of 6×10^4 cells per well, or 35 mm culture dishes containing 2 ml of complete RPMI medium at a density of 12×10^4 cells, and returned to 37 °C, air/CO_2 (19:1) until they were 50–70% confluent.

Molecular biology

The eukaryotic expression vector pMT3 [20] containing the SV40 origin of replication was modified by *Pst*I and *Kpn*I deletion of the multiple cloning site to generate pMT4 [17]. Rabbit SGLT1 cDNA was cloned into the *Eco*RI site of pMT4. The A166C mutation was introduced by PCR mutagenesis as described previously [17].

Construction of N-terminal *myc*-tagged SGLT1

Full-length, blunt-ended SGLT1 cDNA lacking the ATG start codon was generated from SGLT1 cDNA in pMT4 by PCR with cloned *Pfu* DNA polymerase (Stratagene, Aurora, Ontario, Canada). The forward primer was 5′-GACAGCAGCACTTT-GAGCCCC-3′; the reverse primer was 5′-CCTCCAACCAG-CCTGTAATTT-3′. The conditions for PCR were as follows: denaturation at 94 °C for 45 s, followed by 20 cycles of denaturation; annealing at 55 °C for 30 s, extension at 72 °C for 5 min, followed by 72 °C for 40 min. The SGLT1 PCR product was resolved on a 1% (w/v) agarose gel and purified with the Qiagen gel extraction kit. The SGLT1 PCR cDNA sequence was verified by dideoxy chain termination with the Pharmacia T7 polymerase sequencing kit. Before use the PCR product was phosphorylated with T4 polynucleotide kinase (Gibco–BRL, Burlington, Ontario, Canada). SGLT1 PCR product was then blunt-end ligated, in frame, directly behind the *myc*-epitope DNA sequence of vector pNKS2.*myc* [19] (which had been linearized with *Hin*cII), by using T4 DNA ligase (Gibco–BRL). The insert-to-vector ratio was 3:1 and the ligation reaction was performed at room temperature for more than 16 h. Clones containing pNKS2.*myc*-SGLT1 were identified and the orientation of the cDNA insert was verified by *Bam*HI digestion.

Because pNKS2.*myc* does not contain the SV40 origin of replication suitable for expression in the COS-7 cell line, the cDNA coding for the *myc*-tagged SGLT1 was subcloned into vector pMT4. The *myc*-tagged SGLT1 cDNA was removed from pNKS2.*myc* after digestion with *Tsp*509I and ligated into pMT4 digested with *Eco*RI. The correct orientation of the *myc*-tagged SGLT1 cDNA in pMT4 was verified by *Nco*I digestion. Confirmation of the N-terminal tagged *myc*-SGLT1 cDNA sequence

was made by using DNA sequencing with the dideoxynucleotide chain-termination technique [21] as described above.

The DNA used for the COS-7 transfections was prepared from *Escherichia coli* DH5α cells harbouring one of pMT4, pMT4-SGLT1 wild-type, pMT4-SGLT1 A166C or pMT4-*myc*-SGLT1 wild-type by using the Qiagen plasmid midi kit (Qiagen, Chatsworth, CA, U.S.A.). COS-7 cells transfected with vector pMT4, lacking cloned SGLT1 cDNA, served as a control.

Transfection of COS-7 cells with DEAE dextran

COS-7 cells reaching 50–70 % confluency were transfected with the polycationic diethylaminoethyl ether of dextran at a DNA-to-DEAE dextran ratio of 1:40. Chloroquine was used to stabilize the lysosomes during transfection. In brief, medium from the cells was aspirated and replaced with 500 μl of RPMI medium (minus foetal calf serum and antibiotic) containing 40 μg/ml DEAE dextran, 100 μM chloroquine and 1 μg/ml plasmid DNA. Cells were then incubated for 2 h 15 min at 37 °C under air/CO_2 (19:1). After this period the medium was replaced with 500 μl of RPMI medium containing 10 % (v/v) DMSO for 3 min at room temperature. This solution was discarded and 1 ml of complete RPMI medium was added to the cells. They were then returned to 37 °C under air/CO_2 (19:1). Experiments were performed 48–72 h after transfection. The medium was changed when necessary. The above reaction volumes were doubled for cells grown in 35 mm culture wells.

α-MG uptake

The uptake of [^{14}C]α-MG (specific radioactivity 293 mCi/mmol) into COS-7 cells was measured at room temperature (20 °C) as described in [22]. The medium was aspirated from the cells and reactions were started by the addition of 500 μl of incubation medium containing (except where stated) 140 mM NaCl, 20 mM mannitol, 10 mM Hepes/Tris, pH 7.4, and 1 mM α-[^{14}C]MG. After incubation for the desired period the incubation medium was removed and the cells were washed three times with 3 ml of ice-cold stop buffer containing 140 mM KCl, 20 mM mannitol, 10 mM Hepes/Tris, pH 7.4, and 0.2 mM phlorrhizin. The termination and washing procedure took less than 20 s per well. Any remaining solution was aspirated and 500 μl of PBS containing 0.1 % SDS was added to solubilize the cells. After 20 min this solution was removed and processed for liquid-scintillation counting. Where indicated, measurements of transport rates (10 min) were performed. Uptake was directly proportional to time over a period of 20 min.

Phlorrhizin binding assay

The methodology for the measurement of [^{3}H]phlorrhizin binding (specific radioactivity 55 Ci/mmol) was essentially similar to the α-MG uptake assay. The incubation medium contained a specified concentration of phlorrhizin: 0.01, 0.05, 0.1, 0.3, 0.4, 0.5 or 1.0 μM. Measurements of phlorrhizin binding were performed after 1 min. There was no significant increase in the level of phlorrhizin binding after this period. The stop solution did not contain any phlorrhizin. Binding at zero time was not measured.

Estimation of protein

Protein determination was carried out as described by Lowry et al. [23] with the Bio-Rad DC micro-protein assay kit. BSA was used as the standard. The protein concentration of transfected cells cultured in 12-well plates ranged from 0.45 to 0.55 mg/ml per well and from 1.2 to 1.8 mg/ml in 35 mm culture dishes.

Statistical analysis

Results are expressed as means $\pm$ S.D. for n individual experiments, where mean values were determined in triplicate. Tests of significance of difference between mean values was made by using analysis of variance (ANOVA) with a BonFerroni method for multiple comparison t tests between data pairs. Where appropriate, curve fitting was made with a non-linear least-squares-fitting program (Microcal Origin 4.00; Microcal Software, Northampton, MA, U.S.A.).

Materials

α-D-[^{14}C]Glucopyranoside and [^{3}H]phlorrhizin were purchased from Amersham International (Oakville, Ontario, Canada) and Dupont–NEN (Boston, MA, U.S.A.) respectively. DEAE dextran was from Pharmacia (Quebec, Ontario, Canada); chloroquine was from Sigma (St. Louis, MO, U.S.A.). Stock solutions of DEAE dextran (1 mg/ml) and chloroquine (10 mM) were made in RPMI medium without foetal calf serum or antibiotic and kept at -20 °C until use. Phlorrhizin was from Sigma and was prepared as a 100 mM ethanolic concentrated stock solution. The expression vector pMT3 was kindly provided by the Genetics Institute (Boston, MA, U.S.A.). Mouse anti-*c-myc* monoclonal antibody (9E10) was from Berkeley Antibody Company (Richmond, CA, U.S.A.). Goat anti-mouse antibody conjugated to 12 nm colloidal gold was from Jackson, Immunoresearch Laboratories (West Grove, PA, U.S.A.). All other chemicals were of the highest quality available from Sigma.

RESULTS

Localization of SGTL1 expressed in COS-7 cells

Before embarking on detailed functional studies of Na^+-dependent α-MG uptake or phlorrhizin binding, we wanted to be certain that the kinetics of SGLT1 activity in COS-7 cells were due entirely to surface (plasma membrane) expression of the transporter. Accordingly we employed an N-terminal *myc*-tagged SGLT1 mutant to map out its cellular localization by using immunogold labelling and electron microscopy.

The transport properties of this *myc*-tagged isoform were also investigated. In the presence of external 140 mM NaCl there was a significant 6-fold increase in the accumulation of α-MG into COS-7 cells expressing *myc*-tagged SGLT1 compared with those in KCl medium (1.22 ± 0.09 compared with 0.19 ± 0.007 nmol/min per mg of protein respectively; mean $\pm$ S.D., $n = 3$, $P < 0.01$). There was little uptake of α-MG into COS-7 cells transfected with empty plasmid. In the presence of 100 μM phlorrhizin, a competitive inhibitor of SGLT1, Na^+-coupled α-MG transport activity was inhibited by 75 %. The apparent sugar binding affinity was the same for both *myc*-tagged and wild-type protein, K_m values 0.34 ± 0.06 and 0.39 ± 0.08 mM respectively (means $\pm$ S.D. $n = 3$, $P > 0.05$). The maximal rate of transport activity was also not significantly different; the V_{max} values for *myc*-tagged SGLT1 and for wild-type SGLT1 were 2.43 ± 0.39 and 3.00 ± 0.61 nmol/min per mg of protein (means $\pm$ S.D., $n = 3$, $P > 0.05$) respectively. For *myc*-tagged SGLT1 the Na^+-to-glucose coupling stoichiometry was 2:1 and the sugar selectivity sequence (D-glucose > α-MG > D-galactose > 3-*O*-methyl-α-D-glucopyranoside $\gg$ L-glucose) was identical with that of wild-type SGLT1.

Pre-incubation with monoclonal antibody (9E10) [24] (1:100 dilution) did not cause any decrease in α-MG transport activity: 0.62 ± 0.07 compared with 0.66 ± 0.08 nmol/min per mg of protein for cells expressing *myc*-tagged SGLT1 in the presence or absence of the *myc* antibody respectively (means $\pm$ S.D., $n = 3$,

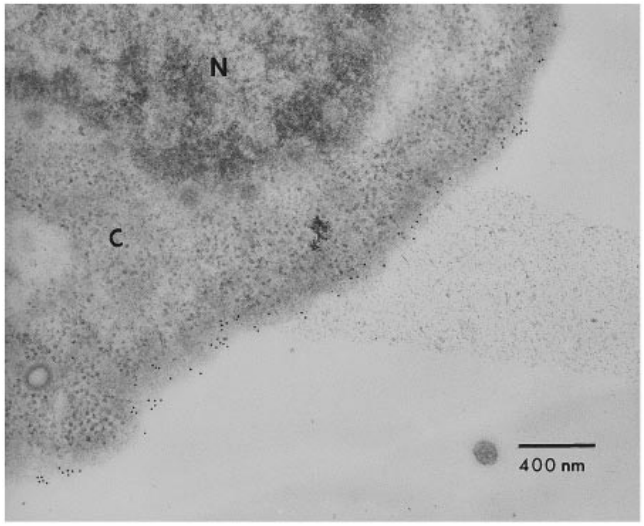

Figure 1 Ultrastructural localization of *myc*-tagged SGLT1 by immunogold labelling of transfected COS-7 cells

COS-7 cells were grown in 35 mm culture dishes and transfected as described in the Materials and methods section. At 48 h after transfection the medium was removed and replaced with 1 ml of RPMI medium (without foetal calf serum or antibiotic) containing monoclonal *myc* antibody (1:100 dilution). The cells were incubated for 1 h at room temperature, washed three times with 1 ml of RPMI medium (10 min each) and treated with goat anti-mouse secondary antibody conjugated to 12 nm colloidal gold particles (1:50 dilution) for 1 h at room temperature. After being washed, the cells were fixed overnight in 2 ml of 0.1 M phosphate buffer, pH 7.2, containing 4% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde. They were then treated with 1% (w/v) osmium tetroxide solution (30 min at room temperature) before ethanol dehydration and embedding with epoxy resin 812 ready for sectioning and transmission electron microscopy. Sections (80 nm thickness) were counterstained with a saturated solution of uranyl acetate for 10 min, then examined and photographed with a Hitachi H600 transmission microscope. Membrane-permeabilizing agents were not used during the immunoreactions. Abbreviations: N, nucleus; C, cytosol.

$P > 0.05$). There was no antibody interaction with cells expressing wild-type SGLT1 (0.76 ± 0.06 compared with 0.71 ± 0.06 nmol/min per mg of protein in the presence or absence of antibody respectively; means $\pm$ S.D., $n = 3$, $P > 0.05$). Similarly there was no effect of the monoclonal antibody on [^{3}H]phlorrhizin binding to either *myc*-tagged SGLT1 or wild-type SGLT1 (results not shown).

Figure 1 shows the ultrastructural localization of an N-terminal *myc*-tagged SGLT1 protein in transiently transfected COS-7 cells and reveals that the protein is localized to the plasma membrane. Moreover, all the gold labelling was external, consistent with the conclusion that the N-terminus of SGLT1 is extracellular. There was no detectable intracellular gold particle labelling and no immunoreactivity with cells expressing wild-type SGLT1 (results not shown).

α-MG uptake

Having demonstrated the ultrastructural localization of SGLT1 in transfected COS-7 cells as being at the cell surface, we next set out to compare the functional properties of SGLT1 A166C with those of wild-type SGLT1. Figure 2 shows the time course of Na^+-dependent α-MG uptake into COS-7 cells transfected with either pMT4-SGLT1 wild-type, pMT4-SGLT1 A166C or pMT4 lacking SGLT1 cDNA. In the absence of Na^+ a small Na^+-independent uptake of α-MG was observed. There was little if any Na^+-dependent uptake of α-MG into COS-7 cells transfected with pMT4. This confirms that COS-7 cells do not possess

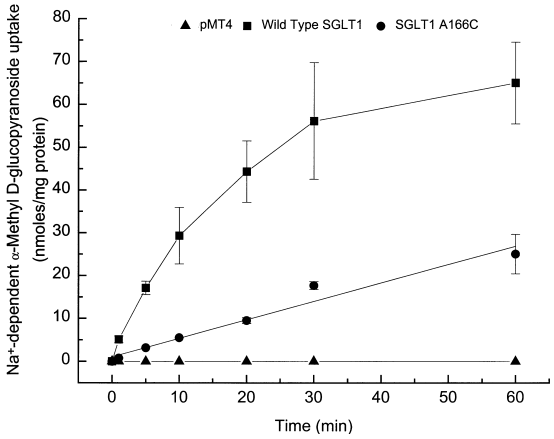

Figure 2 Time course of Na^+-dependent α-MG uptake

COS-7 cells were transfected with pMT4 (▲), pMT4-SGLT1 wild-type (■) or pMT4-SGLT1 A166C (●) and the uptake of α-MG was measured as described in the Materials and methods section. The external medium contained 140 mM NaCl, 20 mM mannitol, 10 mM Hepes/Tris, pH 7.4, and 1 mM α-[^{14}C]MG. The Na^+-independent uptake of 1 mM α-MG was measured in equivalent medium containing 140 mM KCl. For clarity the Na^+-independent uptake values have been subtracted. Results are means $\pm$ S.D. for three separate experiments, each assay performed in triplicate.

endogenous Na^+-dependent glucose transporters. In the presence of external 140 mM NaCl and 1 mM sugar there was a rapid accumulation of α-MG in cells expressing wild-type SGLT1. After 10 min the rate of uptake was significantly greater [(5.3 ± 1.2)-fold] than the rate of α-MG uptake into COS-7 cells expressing SGLT1 A166C (2.92 ± 0.66 compared with 0.55 ± 0.001 nmol/min per mg of protein respectively; $P < 0.01$). The rate of α-MG uptake into cells expressing wild-type SGLT1 continued to increase linearly for approx. 20 min and began to reach a plateau after 30 min. By 60 min the amount of α-MG transported into these cells started to reach equilibrium. In comparison, the uptake of α-MG into cells expressing SGLT1 A166C increased linearly over the same period. Because the number of transporter sites for cells expressing SGLT1 A166C was the same as the number of sites in those expressing wild-type SGLT1 (see Figure 3), the reduced rate of transport activity can account for the fact that equilibrium was not reached after the 60 min period.

The addition of 100 μM phlorrhizin inhibited α-MG uptake into cells expressing either wild-type SGLT1 or SGLT1 A166C by $72 \pm 3\%$ and $75 \pm 7\%$ respectively (means $\pm$ S.D., $n = 3$, $P < 0.01$). Collectively these results indicate that COS-7 cells transfected with pMT4-SGLT1 wild type or pMT4-SGLT1 A166C exhibit functional Na^+-dependent phlorrhizin-sensitive uptake of α-MG. It can also be concluded that the mutation of alanine to cysteine on the rabbit intestinal Na^+/glucose carrier significantly decreased the transport activity of this protein.

Phlorrhizin binding

To determine whether the decrease in α-MG uptake into cells transfected with SGLT1 A166C was due to a decrease in the level of transporter expression, [^{3}H]phlorrhizin binding measurements were performed. COS-7 cells expressing either wild-type SGLT1 or SGLT1 A166C were incubated in reaction medium containing different concentrations of [^{3}H]phlorrhizin (0.01–1 μM). A time course of phlorrhizin binding to SGLT1 expressed in COS-7 cells

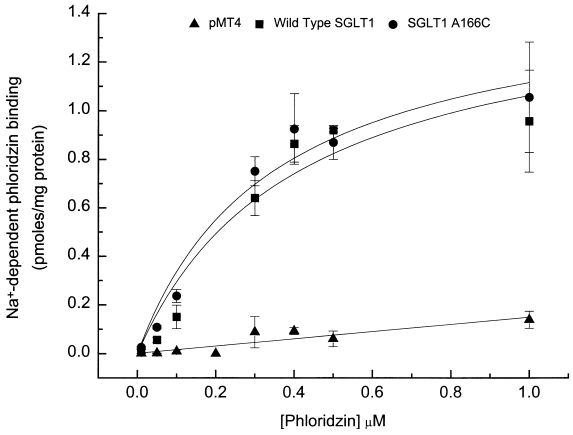

Figure 3 Phlorrhizin binding to transfected COS-7 cells

COS-7 cells were transfected with pMT4 (▲), pMT4-SGLT1 wild-type (■) or pMT4-SGLT1 A166C (●) and the binding of phlorrhizin after 1 min was measured as described in the Materials and methods section. The external medium contained 140 mM NaCl, 20 mM mannitol, 10 mM Hepes/Tris, pH 7.4, and 0.01–1 μM [^{3}H]phlorrhizin. The Na^+-independent uptake of [^{3}H]phlorrhizin was measured in equivalent medium containing 140 mM KCl. For clarity the Na^+-independent uptake values have been subtracted. Results are means $\pm$ S.D. for three separate experiments, each assay performed in triplicate.

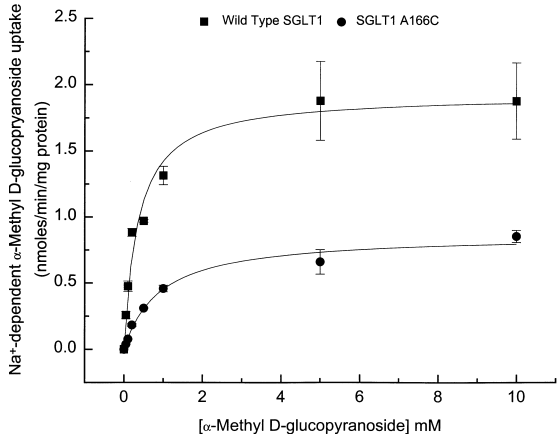

Figure 4 Kinetic parameters

COS-7 cells were transfected with either pMT4-SGLT1 wild-type (■) or pMT4-SGLT1 A166C (●) and the initial rate (10 min) of α-MG transport activity was measured as described in the Materials and methods section. The external medium contained 140 mM NaCl, 10 mM Hepes/Tris, pH 7.4, and 0.05–10 mM α-[^{14}C]MG. The Na^+-independent uptake of α-MG (0.05–10 mM) was measured in equivalent medium containing 140 mM KCl. For clarity the Na^+-independent uptake values have been subtracted. Results are means $\pm$ S.D. for three separate experiments, each assay performed in triplicate.

revealed that maximal equilibrium binding was achieved by 1 min (results not shown). We therefore elected to measure phlorrhizin binding after 1 min in the presence of external 140 mM NaCl or 140 mM KCl. The Na^+-dependent phlorrhizin binding curves (NaCl minus KCl data) are shown in Figure 3.

As the concentration of phlorrhizin increased, the Na^+-dependent binding of ligand also increased. The level of Na^+-dependent phlorrhizin binding to cells transfected with plasmid lacking SGLT1 cDNA was negligible. For both wild-type SGLT1 and SGLT1 A166C, ligand binding increased rapidly over the concentration range 0.01–0.4 μM, approaching saturation at 1 μM. By using a non-linear least-squares-fitting program, the data could be fitted to a rectangular hyperbola, consistent with a single homogeneous phlorrhizin-binding site on SGLT1. Inspection of the binding curves indicates that there was no significant difference between the level of phlorrhizin binding to cells expressing either wild-type SGLT1 or those expressing SGLT1 A166C. The maximal phlorrhizin binding values (B_{max}) were 1.55 ± 0.18 and 1.69 ± 0.57 pmol/mg of protein for SGLT1 A166C and wild-type SGLT1 respectively (means $\pm$ S.D., $n = 3$, $P > 0.05$). Similarly there was no significant difference in the phlorrhizin binding affinities of both SGLT1 isoforms: K_d values were 0.46 ± 0.15 and 0.51 ± 0.11 μM for SGLT1 A166C and wild-type SGLT1 respectively (means $\pm$ S.D., $n = 3$, $P > 0.05$). These values are consistent with phlorrhizin binding measurements performed on vesicles prepared from LLC-PK_1 cells [25], although the B_{max} value was higher in LLC-PK_1 cells (11.6 ± 0.6 pmol/mg of protein). These results support the conclusion that the decreased transport activity is not due to a decrease in the level of SGLT1 A166C expression in COS-7 cells.

Kinetic parameters

Figure 4 shows the kinetic properties of Na^+-dependent α-MG uptake (NaCl minus KCl data) into COS-7 cells expressing either wild-type SGLT1 or SGLT1 A166C. The initial rate of α-MG uptake was estimated by measuring its accumulation at 10 min over a range of substrate concentrations (0.05–10 mM). Both isoforms of SGLT1 expressed in the COS-7 cells exhibited saturating α-MG transport activity, typical of Michaelis–Menten kinetics. Cells expressing SGLT1 A166C showed a significant decrease in the affinity of α-MG (K_m 0.98 ± 0.13 mM) compared with wild-type SGLT1 (K_m 0.36 ± 0.16 mM) ($P < 0.05$). The maximal rate of transport activity was also diminished: V_{max} values were 0.92 ± 0.08 and 1.98 ± 0.47 nmol/min per mg of protein for SGLT1 A166C and wild-type SGLT1 respectively ($P < 0.05$). These results demonstrate that the A166C mutation, relative to wild-type SGLT1, causes a decrease in both the sugar transport affinity and maximal transport rate. Because the phlorrhizin binding results indicate that the number of transporter sites for A166C is the same as wild-type SGLT1 (see Figure 3), the implication of these findings is that the A166C mutation has caused a lowering of transporter turnover rate.

On the assumption that the glucosidic moiety of phlorrhizin binds to the sugar-binding site of SGLT1 [26] and that one molecule of phlorrhizin binds per transporter unit, an estimate of the turnover rate for SGLT1 A166C and wild-type SGLT1 can be calculated. By using the ratio V_{max}/B_{max}, [where V_{max} is the maximal rate of transport activity (pmol/s) and B_{max} is the maximal amount of phlorrhizin binding, i.e. equivalent to the number of Na^+/glucose transporters (pmol/mg of protein)] the turnover rates for SGLT A166C and wild-type SGLT1 expressed in COS-7 cells were calculated to be 9.9 and 19.5 s^{-1} respectively.

Substrate specificity

The sugar specificity of both wild-type and mutant SGLT1 was investigated in competition experiments. Table 1 shows the results of the relative inhibition of 0.1 mM α-MG uptake in the presence of 20 mM sugar analogue. As observed in previous experiments, the rate of α-MG uptake into cells expressing wild-type SGLT1 was (6.6 ± 1.0)-fold the rate of substrate uptake into cells expressing SGLT1 A166C ($P < 0.01$). The order of sugar specificity was D-glucose > α-MG > D-galactose > 3-*O*-methyl-α-D-glucopyranoside $\gg$ L-glucose and was identical for both isoforms of SGLT1.

Table 1 Sugar selectivity sequence

COS-7 cells were transfected with either pMT4-SGLT1 wild-type or pMT4-SGLT1 A166C and the initial rate (10 min) of α-MG transport activity was measured as described in the Materials and methods section. The external media contained 140 mM NaCl, 10 mM Hepes/Tris, pH 7.4, 0.1 mM α-[^{14}C]MG and 20 mM sugar analogue. Results are means ± S.D. for three separate experiments, each assay performed in triplicate. Abbreviation: 3-*O*-MG, 3-*O*-methylglucopyranoside. The control contained 20 mM mannitol.

	α-MG uptake (pmol/min per mg of protein)	
Sugar analogue	Wild-type SGLT1	SGLT1 A166C
Control	1001.14 ± 206.26	150.44 ± 9.94
D-Glc	24.84 ± 2.53	16.66 ± 0.27
α-MG	44.75 ± 1.93	27.98 ± 2.18
D-Gal	46.36 ± 2.42	36.07 ± 3.08
3-*O*-MG	158.06 ± 16.51	77.48 ± 14.57
L-Glc	929.41 ± 49.85	162.57 ± 10.62

DISCUSSION

We have used a *myc*-tagged SGLT1 isoform and an SGLT1 A166C mutant to explore the structure/function properties of these high-affinity Na^+/glucose carriers. The recombinant *myc*-tagged SGLT1 did not affect transporter insertion into the COS-7 cell plasma membrane, kinetics or function, and the immunogold labelling supports previous predictions [5] that the topology of the N-terminal region of SGLT1 is entirely extracellular. That pretreatment of cells expressing *myc*-tagged SGLT1 with monoclonal antibody (9E10) did not impair α-MG uptake or phlorrhizin binding suggests that the epitope sequence is spatially remote from either the Na^+-binding or sugar-binding domains on SGLT1.

So far there has been only one other report on an epitope-tagged SGLT1 protein [27]. This construct was engineered with a 19-residue sequence encoding part of the vesicular stomatitis virus G protein (VSV-G protein) at the C-terminal domain of SGLT1. Although the transporter was successfully expressed in transfected COS-1 cells the introduction of the VSV-G sequence interfered with the kinetic properties of the transporter and possibly its conformation in the membrane [27].

In our previous study of A166C in the oocyte with the two-electrode voltage clamp technique, we documented that the K_m of SGLT1 A166C was increased relative to wild-type SGLT1. By assuming that the charge transfer of both SGLT1 A166C and wild-type SGLT1 was due entirely to the transporter, we calculated that the A166C turnover rate was decreased compared with that of the wild-type carrier. However, without an independent method of quantifying surface expression of SGLT1 A166C and wild-type SGLT1 in the oocyte (e.g. by using freeze–fracture analysis [28]) the full impact of the single cysteine mutation A166C could not be confidently assessed. The COS-7 cell system afforded us the opportunity to extend our functional evaluation of the A166C mutation through performing both Na^+-dependent uptake and phlorrhizin binding measurements.

Having shown that SGLT1 A166C was functionally expressed in the COS-7 cells and had decreased transporter kinetics compared with wild-type SGLT1, we used phlorrhizin binding experiments first to determine that there was equivalent expression of SGLT1 A166C to wild-type SGLT1 in the COS-7 membrane, and secondly to determine whether there was a change in the apparent binding affinity of this ligand to the sugar-binding domain. The N-terminal *myc*-tagged SGLT1 protein provided valuable information that SGLT1 was predominantly localized to the plasma membrane domain of the COS-7 cells and did not show significant internalization. Thus we can be confident that the estimated transporter number from the phlorrhizin binding results is a true reflection of the number of surface-expressed carriers. In addition, because saturating concentrations of external Na^+ were used for these experiments and on the basis of observations in oocytes that the curve of Q against V for SGLT1 A166C shifts slightly to the right, indicating that at −70 mV more than 90% of the transporters are facing outwards [17], the number of Na^+-dependent phlorrhizin binding sites accurately reflect the number of transporters expressed.

There was no significant difference between the number of Na^+-dependent phlorrhizin-binding sites between cells expressing SGLT1 A166C or wild-type SGLT1. Similarly, there was very little difference in the respective phlorrhizin-binding affinities between these isoforms. It was also demonstrated that phlorrhizin binding was inhibited by an excess of α-MG, consistent with the conclusion that the Na^+-dependent component of phlorrhizin binding characterized above represents the binding of phlorrhizin to SGLT1. Thus the equivalent expression of both mutant and wild-type isoforms meant that the decreased α-MG transport activity seen with SGLT1 A166C was not due to defective protein targeting at the plasma membrane.

Competition experiments with a range of D-glucose analogues showed that the hierarchy of sugar specificity was identical for both SGLT1 A166C and wild-type SGLT1, and was identical with that previously observed for rabbit SGLT1 expressed in COS-7 cells [18]. These results, together with the Na^+-dependent phlorrhizin binding results, strongly support the conclusion that the interaction of the sugar with its binding site on SGLT1 A166C is not affected.

The lowering of the apparent affinity (K_m) for SGLT1 A166C expressed in COS-7 cells is consistent with that observed for SGLT1 A166C expressed in oocytes (K_m 0.98 and 0.85 mM respectively) and represents an increase in α-MG K_m of approx. 3-fold. From the phlorrhizin binding and kinetic results in COS-7 cells we were able to calculate the turnover rate for both mutant and wild-type SGLT1 as 9.9 and 19.5 s^{-1} respectively. The values were in good agreement with the turnover rates calculated from the ratio I_{max}/Q_{max} at −70 mV for SGLT A166C and wild-type SGLT1 expressed in oocytes, 8.2 and 17.8 s^{-1} respectively (assuming that two charges per transporter are translocated by the pre-steady-state currents) [17]. In contrast the turnover rate for the SGLT1 mutant D176A [8] was reported to be the same as that of the wild-type transporter, 25 s^{-1} at −150 mV. The quantitative agreement in the A166C turnover rate as determined in the two-electrode voltage-clamp compared with isotope-uptake studies implies that the assumption that the voltage-induced charge transfer in A166C expressed in oocytes is due entirely to the mutant. Thus we have functionally concluded from the above results that the decreased V_{max} of SGLT1 A166C is due to a decrease in the transporter turnover rate. The decreased transport affinity (K_m) for SGLT1 A166C compared with wild-type SGLT1 is currently being evaluated in our laboratory with model simulations for the rate constants for the re-orientation of both the loaded and unloaded carrier (B. Lo and M. Silverman, unpublished work.).

Alanine-166 to cysteine is a conservative substitution, yet the experimental results show that the resultant SGLT1 protein has decreased transport kinetics. Interestingly, a mis-sense mutation, alanine-166 to threonine, was observed in a patient suffering glucose–galactose malabsorption [29]. Expression of this transporter in *Xenopus* oocytes showed significantly less α-MG uptake than controls. Whether the insertion of a more polar amino acid at position 166 causes a decrease in the turnover rate of the

transporter (as described above) or alters the conformation of the transporter owing to changes in electrostatic interactions [8] has not yet been evaluated.

Taken together with previous work from our laboratory on the functional behaviour of A166C, the present study supports the conclusion that the functional alteration caused by this single cysteine mutant is due to an alteration of the interaction of Na^+ with the carrier.

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