Missense mutations in SGLT1 cause glucose–galactose malabsorption by trafficking defects

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Abstract

Glucose–galactose malabsorption (GGM) is an autosomal recessive disorder caused by defects in the Na^+/glucose cotransporter (SGLT1). Neonates present with severe diarrhea while on any diet containing glucose and/or galactose [1]. This study focuses on a patient of Swiss and Dominican descent. All 15 exons of SGLT1 were screened using single stranded conformational polymorphism analyses, and aberrant PCR products were sequenced. Two missense mutations, Gly318Arg and Ala468Val, were identified. SGLT1 mutants were expressed in *Xenopus laevis* oocytes for radiotracer uptake, electrophysiological experiments, and Western blotting. Uptakes of [^{14}C]α-methyl-D-glucoside by the mutants were 5% or less than that of wild-type. Two-electrode voltage-clamp experiments confirmed the transport defects, as no noticeable sugar-induced current could be elicited from either mutant [2]. Western blots of cell protein showed levels of each SGLT1 mutant protein comparable to that of wild-type, and that both were core-glycosylated. Presteady-state current measurements indicated an absence of SGLT1 in the plasma membrane. We suggest that the compound heterozygote missense mutations G318R and A468V lead to GGM in this patient by defective trafficking of mutant proteins from the endoplasmic reticulum to the plasma membrane. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Glucose–galactose malabsorption; Human Na^+/glucose cotransporter 1 (hSGLT1); Protein trafficking

1. Introduction

Cotransporters are a major class of integral membrane proteins responsible for the absorption of various solutes across the plasma membrane; they concentrate substrate molecules into cells by harnessing the energy from the electrochemical gradient of Na^+ or H^+ ions. More specifically, the Na^+/glucose cotransporter (SGLT1) couples the transport of Na^+ and glucose across the brush border of the small intestine. Glucose–galactose malabsorption (GGM) is an autosomal recessive disorder caused by defects of SGLT1 [3]. Congenital defects result in severe diarrhea and dehydration in the newborn and, if not diagnosed and treated, the condition can be fatal. On the other hand, if appropriate dietary carbohydrate restriction is applied, the diarrhea stops and the child develops normally.

To date, we have identified 20 missense mutations of the SGLT1 gene leading to reduction or elimina-

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tion of transport activity [4–6]. However, only the mutations identified in two patients have been studied in any significant detail. Here we report the identification of two newly recognized mutations in a GGM patient. Using established techniques and an oocyte expression system, we identified specific mutations of each allele, and examined how these mutations cause the defect in sugar transport. The results suggest that both mutant SGLT1 proteins fail to correctly traffic from the endoplasmic reticulum to the plasma membrane.

2. Materials and methods

2.1. Patient background

The proband, C.S., was the 3520-g product of a normal full-term pregnancy born to a mother of Swiss, and a father of Dominican descent. Her diet consisted of both a dextrin–maltose-based formula and breast milk. After 48 h of life, her stools became progressively frequent and watery, and she was hospitalized with life-threatening hypernatriemic dehydration. After discontinuing the enteral diet, her diarrhea ceased, and subsequent attempts to introduce lactose, dextrin–maltose or glucose containing foods resulted in further bouts of diarrhea. A carbohydrate-free formula (Nutricia, The Netherlands) with added fructose was started, and she has thrived since without further episodes of diarrhea. At age 2.5 years she tolerated at least 1.25 g ($\sim$100 mg/kg b.wt.) of glucose per day in the form of starch, fruits, and vegetables.

At age 5 months, galactose malabsorption was documented by an oral loading test (1 g galactose/kg b.wt.) after which blood galactose remained unmeasurable and blood glucose unchanged [7]. At age 7 months, several biopsies of the small intestine were taken. Control biopsies were taken from the same region of the duodenum of a celiac disease patient in remission, as proven by histology. Aliquots were used for in vitro studies of monosaccharide accumulation. The 1-mM glucose accrual factor (tissue to medium concentration ratio after 1 h) was 2.8 for glucose (control 5.3) and 2.7 for galactose (control 7.3). These results confirmed the clinical diagnosis and corresponded to published values [8].

We investigated the distribution of SGLT1 by immunocytochemistry staining. Other aliquots of the biopsies were fixed in Carnoy's solution, rinsed with phosphate-buffered saline, and stored in 20% (w/v) buffered sucrose. Using a SGLT1 specific antibody we were able to identified the location of the transporters in the duodenum [9,10].

2.2. Molecular biology

Genomic DNA was extracted from the whole blood sample and used to amplify all 15 exons and 527 base pairs of the promoter of SGLT1 by polymerase chain reaction (PCR) using intron-based oligonucleotides flanking each exon. The oligonucleotides and the basic methods used are similar to those described previously [5]. Each exon was screened against the wild-type product by single-stranded conformational polymorphism (SSCP) analysis. The heat-denatured and labeled PCR product was electrophoresed on a non-denaturing Mutation Detection Enhancement gel (FMC Bioproducts, Rockland, ME) for 16 h at 4 W at room temperature. PCR products with aberrant migration pattern were again amplified by PCR without isotope, gel purified, and PCR products sequenced bidirectionally using standard methods to identify the mutations [4,11].

To study the effect of the mutation, site-directed mutagenesis was used to synthesize the mutant SGLT1 clones [12]. The 'mutagenizing' oligonucleotides used are listed below with the bold letters indicating the mutated nucleotide: G318R-sense, 5′-GCA TCC TGT G**T**A GGT ATC TAA AGC-3′, G318R-antisense, 5′-GCT ATT GAT ACC **TAC** ACA GGA TGC-3′; A468V-sense, 5′-GGG ACC ACC CAT G**T**T GGC TGT CTT CC-3′; and A468V-antisense, 5′-GGA AGA CAG CCA **A**CA TGG GTG GTC CC-3′. The mutant Bluescript plasmids were linearized with *Xba*I, treated with proteinase K, extracted with phenol/cholorform, and precipitated with 2-propanol. In vitro transcription was performed by a standard protocol using MEGA-Script T3 Kit from Ambion (Austin, TX).

2.3. Radiotracer uptake and Western blot analysis

Mature oocytes were harvested from *Xenopus laevis* and injected with 50 ng of capped cRNA for

SGLT1 coding for human wild-type and the mutants G318R and A468V. Oocytes were then incubated at 18°C in Barth's medium for 5–14 days. Sugar transport was assayed by a radiotracer method. Groups of 8–12 oocytes were incubated for 1 h at 22°C in 500 μl of Na$^+$ buffer containing 50 μM [^{14}C]methyl-D-glucoside (α-MDG) (2 μCi/ml) [13].

Injected oocytes were also solubilized in 100 mM NaCl, 20 mM Tris HCl (pH 7.5), and 1% Elugent (Calbiochem, CA), and centrifuged to remove insoluble yolk and lipids. Samples were then mixed with SDS loading buffer (2% sodium dodecyl sulfate, 63 mM Tris HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and run on a 9% Tricine/SDS polyacrylamide electrophoresis mini gel. Separated proteins were electrotransferred to nitrocellulose membrane. SGLT1 was detected using an antibody raised to peptide residues 602–613 in the C-terminal portion of SGLT1 at a dilution of 1:3000 [14].

2.4. Electrophysiology

A two-microelectrode voltage clamp was used to measure wild-type and mutant Na$^+$/glucose cotransporter expression and kinetics in oocytes [15,16]. Currents measured at different sugar concentrations were fitted to the equation, $I = I_{max} [S]^n/((K_{0.5})^n + [S]^n)$, where I_{max} is maximum current, n is the Hill coefficient, S is the sugar concentration, and $K_{0.5}$ is the sugar concentration that produces $I_{max}/2$. These sugar-dependent inward Na currents are directly proportional to sugar uptakes [2]. Membrane potential was stepped to various voltages ranging from −150 to 50 mV and pre-steady-state currents were obtained by subtracting the capacitive component and steady-state currents from the total membrane current using a standard protocol. Charge movement (Q) was obtained by the integration of the transient current recorded in the absence of sugar after each test potential [15]. Q/V plots were fitted to the Boltzman equation and the total number of transporters in the plasma membrane was estimated from $N = Q_{max}/z \cdot e$, where z is the valence of SGLT1, e is the elementary charge of an electron, and N is the estimated number of transporters in the plasma membrane [15,16]. Using freeze-fracture electron microcopy we have previously demonstrated the validity of this method to determine the number of wild-type and mutant SGLT1 proteins in the oocyte plasma membrane [6,10,17]. pClamp software (Axon Instruments, Foster City, CA) was used to control the pulse protocol.

3. Results

3.1. Mutation analysis

Only exons 5, 7, 10, and 15 had a normal migration pattern by SSCP analysis, therefore the remaining exons and promoter region were sequenced bidirectionally. Mutations were identified only in exons 9 and 12. In exon 9, a glycine at residue 318 was changed to an arginine (G318R) by a single nucleotide substitution of (GGG→AGG) on one allele. Similarly, in exon 12, a single nucleotide substitution of (GCG→GTG) at residue 468 resulted in a change of an alanine to a valine (A468V) as shown in Fig. 1. This mutation also resulted in the elimination of a *Bsr*DI site. With restriction enzyme digestion of parental and patient DNA, we confirmed the presence of the mutation and that the A468V mutation was inherited from the mother, suggesting that the father
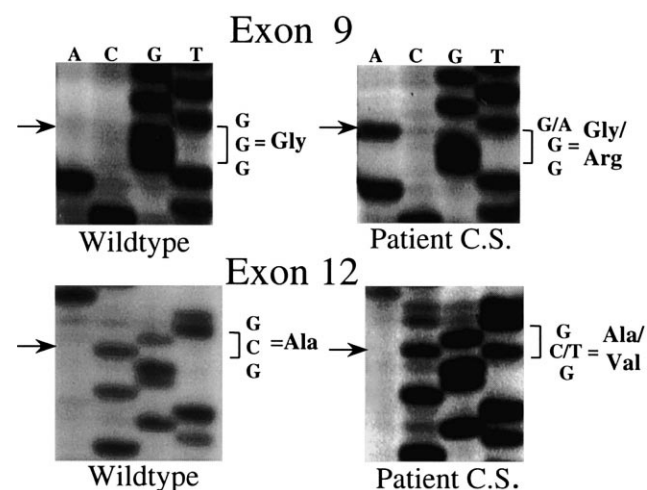

Fig. 1. Analysis by SSCP and PCR sequencing of the 15 exons of hSGLT1 identified two novel missense mutations in exons 9 and 12 in the compound heterozygous patient. In exon 9 of one allele, a single nucleotide substitution of GGG→AGG at residue 318 mutated a glycine to an arginine, in exon 12 of the other allele, substitution of GCG→GTG at residue 468 resulted in a change of an alanine to a valine.
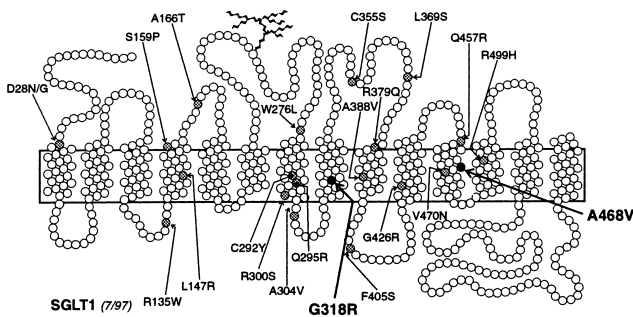
Fig. 2. The locations of GGM missense mutations on the schematic secondary structure of hSGLT1. Highlighted circles denote the substitution at the specified amino acid residues. G318R and A468V are located in the transmembrane mutation 'hot spots' between exons 9 and 12. The mutations of the proband are highlighted.

was the donor of the G318R mutation. Fig. 2 shows that both mutations identified reside in transmembrane domains of the secondary structure; G318R and A468V are located in the eighth and eleventh transmembrane spans, respectively [18]. No other mutations were found in other exons by additional sequencing.

3.2. Glucose uptake and Western analysis

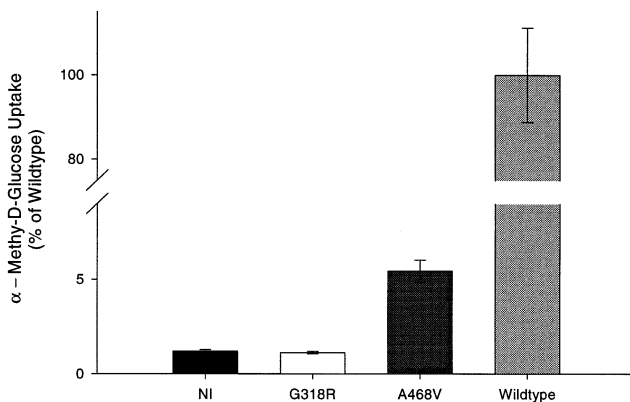To assess if there are any changes in function due to the mutations identified, the oocyte expression system was used to measure the uptake of radiolabeled sugar as compared to that of the wild-type. The [^{14}C]α-MDG uptake of G318R SGLT1 was indistinguishable from non-injected oocytes, (i.e. < 1% of that of the wild-type) while the uptake for the A468V SGLT1 was 5% that of wild-type (Fig. 3).

Western blot analysis of oocyte protein demonstrated that SGLT1 protein synthesis was comparable in wild-type, G318R, and A468V SGLT1 expressing oocytes. The intensity of the ~60-kDa immunoreactive SGLT1 band was approximately the same in all oocytes, but in those expressing wild-type SGLT1, there was a second immunoreactive band with an apparent molecular weight of ~70 kDa (Fig. 4). The 60-kDa protein represents the core-glycosylated SGLT1 and the 70-kDa protein, the fully glycosylated protein [10]. It should be noted that glycosylation is not required for the functional expression of SGLT1 in oocytes, or the activity of SGLT1 in brush-border membranes [19,20].

3.3. Electrophysiology

The expressed proteins were studied further by using electrophysiological techniques. Human wild-type SGLT1 showed phlorizin-sensitive, sodium-de-

Fig. 3. [^{14}C]α-MDG radiotracer uptake by *Xenopus laevis* oocytes injected with 50 ng of cRNA coding for wild-type, mutant G318R, or A468V hSGLT1. Uptake in 50 μM [^{14}C]α-MDG is represented by the mean of 12 oocytes. Wild-type hSGLT1 sugar uptake was 366±40 pmol/oocyte/h. G318R sugar uptake was indistinguishable from that of non-injected oocytes (4.6±0.25 pmol/oocyte/h), while A468V uptake was 5-fold higher (20±2.1 pmol/oocyte/h).
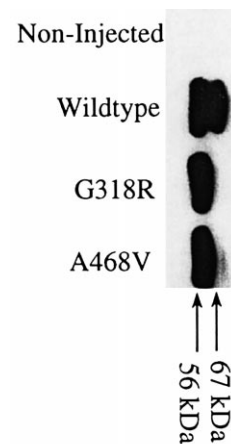

Fig. 4. Western blot analysis of oocytes expressing wild-type, G318R, or A468V hSGLT1. Protein synthesis was not interrupted by the mutation. The lower band at ~60 kDa is present in all samples in equal intensity and represents the core glycosylated protein. The upper band at ~70 kDa in wild-type hSGLT1 corresponds to the complex glycosylated protein. G318R lacks the upper band completely and A468V has a band of very low intensity at that location (<1% in intensity of wild-type).
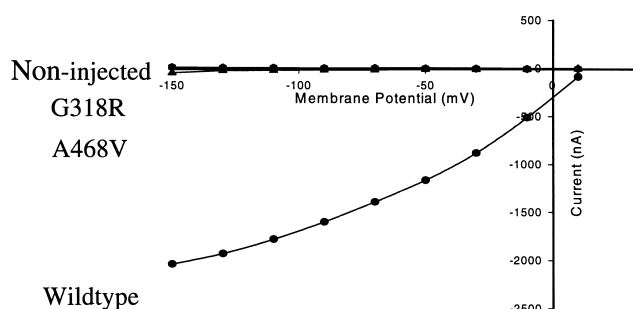
Fig. 5. Steady-state currents produced by wild-type and mutant hSGLT1. The sugar-induced current produced by wild-type at -150 mV was 1900 nA, whereas no noticeable currents were induced by G318R or A468V. Injected oocytes expressing wild-type, G318R, or A468V were incubated in 100 mM NaCl buffer and the difference in steady-state currents between the presence and absence of 10 mM α-MDG were plotted at each test membrane potential.

pendent, sugar-induced Na^+ currents with a α-MDG $K_{0.5}$ of 0.9–1.1 mM. The maximum sugar current at -150 mV was 1900 nA, whereas for the mutant SGLT1s, there was no noticeable sugar-induced current for the entire voltage range tested (Fig. 5).

Integration of the transient currents obtained with each voltage jump provided the charge transfer, Q. Using SGLT1 charge movement, we calculated the number of co-transporters in the plasma membrane. When the wild-type Q/V curve was fitted to the

Boltzman equation with a Q_{max} of 37 nC, the total number of co-transporters was determined to be 1.2×10^{11} transporters. In the case of the mutants, there was no significant level of charge movements (Fig. 6), and this suggests that the mutant cotransporters were not inserted into the oocyte plasma membrane (i.e. less than 10% of the wild-type co-transporters). To determine if this was also the case in the patient's enterocytes we carried out SGLT1 immunocytochemistry on intestinal biopsies obtained from the GGM patient and controls. There was a marked reduction in SGLT1 immunoreactivity in the patient's enterocytes, and unlike the normal tissue where immunoreactivity was restricted to the brush border, intracellular staining was observed between the nuclei and the brush-border membrane (B. Hirayama and M. Lostao, personal communication) [10].

4. Discussion

A major research focus of this laboratory has been the mechanism of Na^+ and glucose transport across plasma membranes by SGLT1. One approach is to identify naturally occurring mutations in the SGLT1 gene in GGM patients. We previously identified 20
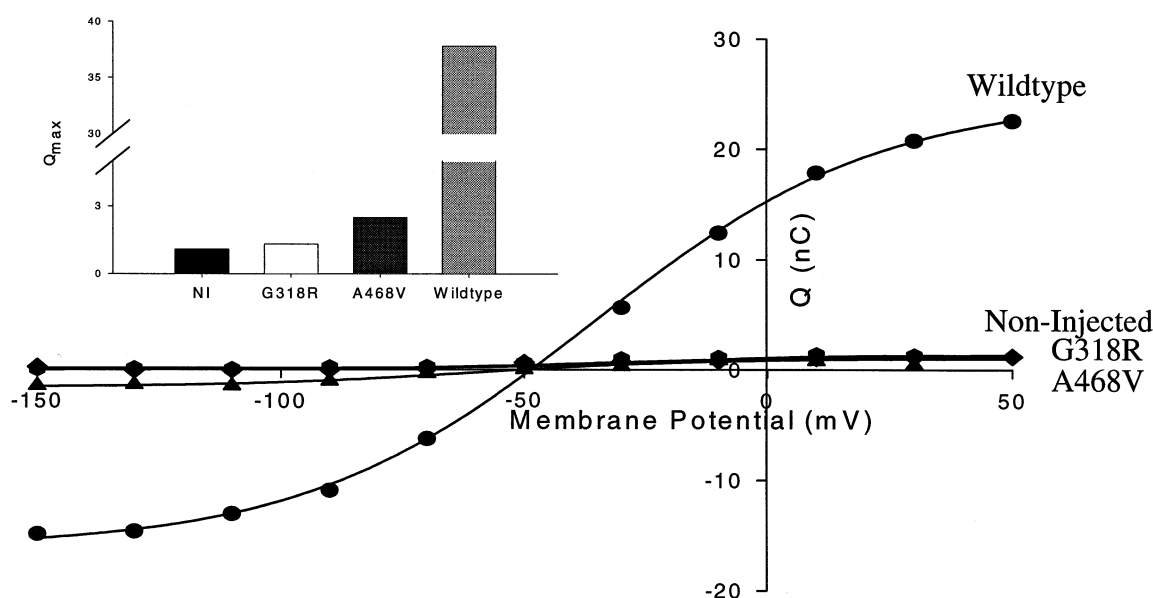

Fig. 6. Charge movement in injected oocytes expressing wild-type and mutant hSGLT1. Wild-type shows a Q_{max} of 37 nC which correspond to 1.2×10^{11} transporters in the plasma membrane per cell. G318R shows charge movement indistinguishable from non-injected cells while A468V shows a Q_{max} twice that of background.

missense mutations in GGM patients that account for severely impaired Na^+/glucose transport [4–6]. The most frequent effect of these mutations was to reduce the number of SGLT1 transporters in the plasma membrane. In this study, we report on the identification of two additional missense mutations, G318R and A468V, that impair sugar transport by reducing the number of cotransporters trafficked to the plasma membrane. G318 and A468 are well conserved within the large SGLT1 gene family (see [21]). Both mutations resulted in at least a 95% reduction of sugar transport as evident from the $[^{14}C]\alpha$-MDG uptakes and sugar dependent sodium currents in oocytes (Figs. 3 and 5). Moreover, the SGLT1 charge measurements (Q_{max}) indicate that the number of SGLT1 proteins in the plasma membrane was substantially smaller in oocytes injected with mutant cRNAs, less than 10% of wild-type. We have previously established the validity of charge measurements to estimate the number of wild-type and mutant SGLT1 transporters in the plasma membrane of oocytes – Q_{max} is directly proportional to the number of SGLT1 intramembrane particles in the plasma membrane [6,10,17]. The effective reduction in number of transporters was not due to a defect in SGLT1 synthesis or stability since Western blots of cell protein showed comparable levels of SGLT1 in oocytes injected with the wild-type or mutant SGLT1 cRNAs (Fig. 4). However, there was little or no complex glycosylation of the mutant proteins, suggesting that the mutations caused a defect in translocation of SGLT1 between the ER and the Golgi apparatus. These results indicate that with these two new missense mutations the defect in sugar transport is due to a problem in SGLT1 trafficking.

Evidence is rapidly accumulating that many disease-causing mutations are due to improper trafficking; these diseases include cystic fibrosis, retinitis pigmentosa, and leprechaunism [22]. In the case of GGM, the studies of 22 missense mutant SGLT1 proteins in oocytes indicate that this disease is primarily due to improper trafficking. These mutations are distributed throughout the 664 residue protein: four are in the external hydrophilic loops, three are in the cytoplasmic hydrophilic loops, and 15 are within the membrane domain. The majority of the mutations involve charged or polar residues, e.g. G318R in transmembrane helix 8 (TMH8), where it

is anticipated that the charged residue will grossly destabilize the structure. It is noteworthy that three are mutations from alanine to valine (A304V in the intracellular loop between TMH7 and TMH8; A388V TMH9; and A468V TMH11), suggesting that changes in bulk at critical domains in the protein are sufficient to cause conformational changes that alter trafficking. Preliminary studies where A468 was mutated to 468C, support this notion [23]. The molecular volume of cysteine (108.5 $\AA^3$) is intermediate between that for alanine (88.6 $\AA^3$) and valine (140 $\AA^3$). The A468C SGLT1 protein was functional, the maximum rate of transport was 25% of that for wild-type and the glucose $K_{0.5}$ was in the normal range (0.8 mM). These results suggest that simply increasing the bulk of residue 468 from alanine to cysteine to valine in TMH11 causes graded changes in the 3-D conformation of SGLT1 that in turn produce graded perturbations in trafficking of the cotransporter to the plasma membrane.

Finally, eight of 29 GGM patients are compound heterozygotes; they include both nonsense and splice-site mutations as well as missense mutations found in the present case. The fact that 30% of the GGM patients studied are the result of non-consanguineous relationships suggests that there is a pool of people who are carriers for GGM mutations. Whether or not these heterozygotes have any impaired glucose absorption has yet to be determined, but it is noted that about 10% of the normal population, medical students, showed glucose malabsorption with H_2-breath tests [24].

Acknowledgements

References

[1] J.-F. Desjeux, E. Turk, E.M. Wright, Congenital selective Na^+ D-glucose cotransport defects leading to renal glycosu-

ria and congenital selective intestinal malabsorption of glucose and galactose, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), Metabolic Basis of Inherited Disease, 7th edn., McGraw-Hill, New York, 1995, pp. 3563–3580.

[2] B. Mackenzie, D.D.F. Loo, E.M. Wright, Relations between Na$^+$/glucose cotransporter (SGLT1) currents and fluxes, J. Membr. Biol. 162 (1998) 101–106.

[3] V.A. McKusick, Mendelian Inheritance in Man: Catalogs of Human Genes and Genetic disorders, 12th edn., Johns Hopkins University Press, Baltimore, MD, 1998. Available also as 'On-line Mendelian Inheritance in Man (OMIM)' (http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html); OMIM # 182380.

[4] E. Turk, B. Zabel, S. Mundlos, J. Dyer, E.M. Wright, Glucose/galactose malabsorption caused by a defect in the Na$^+$/glucose cotransporter, Nature 350 (1991) 354–356.

[5] M.G. Martín, E. Turk, M.P. Lostao, C. Kerner, E.M. Wright, Defects in Na$^+$/glucose cotransporter (SGLT1) trafficking and function cause glucose–galactose malabsorption, Nat. Genet. 12 (1996) 216–220.

[6] M.G. Martín, M.P. Lostao, E. Turk, J. Lam, M. Kreman, E.M. Wright, Compound missense mutations in the sodium/D-glucose cotransporter (SGLT1) results in trafficking defects, Gastroenterology 112 (1997) 1206–1212.

[7] G. Kurz, K. Wallensfels, D-Galactose-UV-Test mit Galactose-Dehydrogenase, in: H.U. Bergmeyer (Ed.), Methoden der Enzymatischen Analyse, Vol. 2, Verlag Chemie, Weinheim, Germany, 1970, pp. 1241–1244.

[8] L. Evans, E. Grasset, M. Heyman, A.M. Dumontier, J.P. Beau, J.F. Desjeux, Congenital selective malabsorption of glucose and galactose, J. Pediatr. Gastroenterol. Nutr. 4 (1985) 878–886.

[9] K. Takata, T. Kasahara, M. Kasahara, O. Ezaki, H. Hirano, Immunolocalization of glucose transport GLUT1 in the rat placental barrier: possible role of GLUT1 and the gap junction I the transport of glucose across the placental barrier, Cell Tissue Res. 267 (1992) 3–9.

[10] M.P. Lostao, B.A. Hirayama, M. Panayotova-Heiermann, S.L. Sampogna, D. Bok, E.M. Wright, Arginine-427 in the Na/glucose cotransporter (SGLT1) is involved in trafficking to the plasma membrane, FEBS Lett. 377 (1995) 181–184.

[11] M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction, Genomics 5 (1989) 874–879.

[12] R.B. Higuchi, B. Krummel, R.K. Saiki, A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, Nucleic Acids Res. 16 (1986) 7351–7367.

[13] T.S. Ikeda, E.-S. Hwang, M.J. Coady, B.A. Hirayama, M.A. Hediger, E.M. Wright, Characterization of a Na$^+$/glucose cotransporter cloned from rabbit small intestine, J. Membr. Biol. 110 (1989) 87–95.

[14] B.A. Hirayama, H.C. Wong, C.D. Smith, B.A. Hagenbuch, M.A. Hediger, E.M. Wright, Intestinal and renal Na/glucose cotransporters share common structures, Am. J. Physiol. 261 (1991) C296–C304.

[15] D.D.F. Loo, A. Hazama, S. Supplisson, E. Turk, E.M. Wright, Relaxation kinetics of the Na$^+$/glucose cotransporter, Proc. Natl. Acad. Sci USA 90 (1993) 5767–5771.

[16] L. Parent, S. Supplisson, D.F. Loo, E.M. Wright, Electrogenic properties of the cloned Na$^+$/glucose cotransporter. Part I. Voltage-clamp studies, J. Membr. Biol. 125 (1992) 49–62.

[17] G.A. Zampighi, M. Kreman, K.J. Boorer, D.D.F. Loo, F. Bezanilla, G. Chandi, J.E. Hall, E.M. Wright, A method for determining the unitary functional capacity of cloned channels and transporters expressed in *Xenopus laevis* oocytes, J. Membr. Biol. 1148 (1995) 65–79.

[18] E. Turk, C.J. Kerner, M.P. Lostao, E.M. Wright, Membrane topology of the human Na$^+$/glucose cotransporter SGLT1, J. Biol. Chem. 271 (1996) 1925–1934.

[19] M.A. Hediger, J. Mendlein, H.-S. Lee, E.M. Wright, Biosynthesis of the cloned Na$^+$/glucose cotransporter, Biochim. Biophys. Acta 1064 (1991) 360–364.

[20] B.A. Hirayama, E.M. Wright, Glycosylation of the rabbit intestinal brush border Na$^+$/glucose cotransporter, Biochim. Biophys. Acta 1103 (1992) 37–44.

[21] E. Turk, E.M. Wright, Membrane topological motifs in the SGLT cotransporter family, J. Membr. Biol. 159 (1997) 1–20.

[22] P.J. Thomas, B.H. Qu, Pedersen, P.L. Defective protein folding as a basis of human disease, Trends Biochem. Sci. 20 (1995) 441–488.

[23] E.M. Gallarado, B.A. Hirayama, D.D.F. Loo, J.T. Lam, E. Turk, E.M. Wright, Identification of the sugar translocation pathway of the Na$^+$/glucose cotransporter (SGLT1), FASEB J. 12, (5) (1998) A1017.

[24] R.G. Montes, R.G. Gottal, T.M. Bayless, T.R. Hendrix, J.A. Perman, Breath hydrogen testing as a physiology laboratory exercise for medical students, Am. J. Physiol. 262 (1992) S25–S28.