A missense mutation in the Na\(^+\)/glucose cotransporter gene SGLT1 in a patient with congenital glucose-galactose malabsorption: normal trafficking but inactivation of the mutant protein

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Abstract

The Na\(^+\)/glucose cotransporter gene SGLT1 was analyzed in a Japanese patient with congenital glucose-galactose malabsorption. Genomic DNA was used as a template for amplification by the polymerase chain reaction of each of the 15 exons of SGLT1. The amplification products were cloned and sequenced. About half of the exon 5 clones contained a C\(\rightarrow\)T transition, resulting in an Arg 135 \(\rightarrow\) Trp mutation, whereas the remaining clones contained the normal exon 5 sequence. In addition, whereas some exon 12 clones exhibited the normal sequence, others showed a CAG\(\rightarrow\)CAT mutation at the splice donor site of intron 12 that may result either in the skipping of exon 12 or in read-through of intron 12. Neither the Arg\(^{135}\) \(\rightarrow\) Trp mutant nor either of the possible intron 12 mutant proteins exhibited Na\(^+\)-dependent glucose transport activity when expressed in \textit{Xenopus} oocytes. Immunocytochemical analysis indicated, however, that the Arg\(^{135}\) \(\rightarrow\) Trp mutant was localized to the oocyte plasma membrane. DNA sequence analysis revealed that the missense mutation in exon 5 and the splice site mutation in intron 12 were inherited from the proband’s father and mother, respectively. These results indicate that the patient is a compound heterozygote for this disease, and that the Arg\(^{135}\) \(\rightarrow\) Trp mutant of SGLT1 undergoes normal trafficking to the plasma membrane but is non-functional. ß 2001 Elsevier Science B.V. All rights reserved.

Keywords: Na\(^+\)/glucose cotransporter; SGLT1; Congenital glucose-galactose malabsorption; Protein trafficking

1. Introduction

Absorption of monosaccharides in the small intestine of mammals is mediated by specific transporters in the apical membrane of epithelial cells. Glucose and galactose are actively transported into these cells by the Na\(^+\)-dependent glucose transporter SGLT1 [1], whereas fructose is transported by Glut5 [2] or Glut2 [3]. The human SGLT1 gene is located on chromosome 22q13.1, comprises 15 exons, and encodes a 73-kDa hydrophobic protein predicted to contain 14 transmembrane segments [1].

Congenital glucose-galactose malabsorption (GGM), which was first described in 1962 [4,5], is
an autosomal recessive disease characterized by the selective malabsorption of glucose and galactose, with the absorption of fructose being unimpaired [6,7]. Only ~200 individuals worldwide are currently known to be affected by GGM [7], five of whom reside in Japan [8,9]. We have now studied one of these Japanese patients and shown that she is a compound heterozygote for defects in \textit{SGLT1}, exhibiting a missense mutation in exon 5 and a splice site mutation in intron 12. The protein affected by the missense mutation in exon 5 exhibited normal trafficking to the plasma membrane but showed little transport activity, possibly because of a change in its conformation in the membrane.

2. Materials and methods

2.1. Patient background

The clinical course of the patient will be described in detail elsewhere. In brief, the proband was the 3234-g product of a normal full-term pregnancy born to Japanese parents in 1994. Breast feeding began on the day following birth and frequent watery diarrhea became noticeable on day 3. On day 7, she was admitted to the neonatal intensive care unit of Fukui Red Cross Hospital with symptoms of emaciation, apathy, and severe dehydration and a body weight of 2674 g. The concentrations of analytes in serum on admission were as follows: Na\(^+\), 174 mEq/l; K\(^+\), 4.9 mEq/l; Cl\(^-\), 145 mEq/l; urea nitrogen, 39.7 mg/dl; creatine, 0.8 mg/l. Reducing sugars were found in acidic feces (pH 5.5). The hypertonic dehydration was treated with parenteral fluid therapy. The diarrhea stopped with the cessation of breast feeding and started again when breast feeding was resumed. The patient’s condition did not change on a lactose-free formula in which lactose was replaced with glucose and polysaccharides, nor on 5% glucose solution. The introduction of fructose-supplemented, carbohydrate-free formula resulted in a marked improvement in her diarrhea. She grew and developed normally while continuing with the fructose-supplemented formula, and was discharged from the hospital in a healthy state at age 49 days. On the basis of her clinical course, she was diagnosed with GGM.

2.2. Molecular biology

Genomic DNA was extracted from whole blood with the use of an extraction kit (Takara) and used as the template for amplification of all 15 exons of \textit{SGLT1} by the polymerase chain reaction (PCR). The primers (forward and reverse, respectively) were as follows: 5’-CACATCGCAGGACAGCTTT and 5’-TAAAGTGTCCTCAGTATT (exon 1); 5’-TGCTGGCAGGCGGCT and 5’-GGATTATTTACTGTTTG (exon 2); 5’-CAGGTCCTGTTGTGGCCTCAGTAT and 5’-ACCTGCTGGAAGCCAAAT (exon 3); 5’-GACCTGCTGTTGGCCGACAT and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-AATCAGGAGTAGAGAGACTA and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-AATCAGGAGTAGAGAGACTA and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-GACCTGCTGTTGGCCGACAT and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-AATCAGGAGTAGAGAGACTA and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-AATCAGGAGTAGAGAGACTA and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-GACCTGCTGTTGGCCGACAT and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5); 5’-AATCAGGAGTAGAGAGACTA and 5’-GGGTTTGAGGTGCTGGTTTA (exon 5). For expression of human SGLT1 in \textit{Xenopus} oocytes, pBSXG (pBluescript KS(+) containing the \textit{Xenopus} \textit{L}-globin gene promoter and terminator) [10] was modified by deletion of the sequence between the \textit{HincII} and \textit{ ClaI} sites in the multicloning site region and insertion of an \textit{XbaI}–\textit{NotI}–\textit{ClaI} linker into the \textit{BglII} site. The human \textit{SGLT1} cDNA was
inserted into the modified plasmid with the use of XbaI and Clai. The Arg^{135}→Trp mutation was introduced into SGLT1 with the use of megaprimer PCR mutagenesis [11,12]. For generation of the exon 12-skipping form of SGLT1, the primer 5’-GTTGTTAACCCATCATCGATTAGTCCCCAA-AAGGCTCCCTCAGGACATCA was used, resulting in a five-amino acid extension before reaching a stop codon. For generation of the intron 12-read-through form of SGLT1, the primer 5’-GTGGTAAACCAGATCATCGATTATGCCCAGATGAC was used, resulting in an 11-amino acid extension before reaching a stop codon. Plasmids were digested with PstI, and mRNAs were prepared by in vitro transcription (T7 mMESSAGE mMACHINE; Ambion).

2.3. Transport assay and immunoblot analysis

Oocytes at stage 5 or 6 were harvested from Xenopus laevis females and injected with 50 nl (~50 ng) of mRNA encoding wild-type or mutant human SGLT1. They were then cultured for 3–5 days at 18°C in a medium containing 88 mM NaCl, 1.1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.81 mM MgSO₄, 15 mM HEPES–NaOH (pH 7.6), penicillin (1000 U/ml), and streptomycin (100 µg/ml) [13]. Glucose transport activity in groups of three oocytes was assessed by measurement of the uptake of 50 µM [¹⁴C]methyl-D-glucose (CFB 76, Amersham Pharmacia Biotech) in an uptake solution containing 100 mM NaCl, 2 mM KCl, 2 mM MgCl₂, and 10 mM HEPES–Tris (pH 7.5). After incubation at 20°C for 1 h, oocytes were immediately washed three times with 2 ml of ice-cold uptake solution. Individual oocytes were lysed in 200 µl of 10% SDS, and the amount of released radioactivity was determined by a liquid scintillation counter.

For immunoblot analysis, groups of five oocytes were homogenized, and the homogenate was centrifuged at 1000×g for 30 s to eliminate yolk proteins. The resulting supernatant was subjected to SDS–PAGE, followed by immunoblot analysis with rabbit polyclonal antibody to the peptide corresponding to amino acids 564–575 of the deduced SGLT1 sequence [14] and [¹²⁵I]-labeled protein A [15].

2.4. Immunocytochemistry

Oocytes incubated for 3–5 days after mRNA injection were fixed in 4% paraformaldehyde for 2 h at room temperature, washed three times with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM sodium phosphate–1.5 mM potassium phosphate, pH 7.5), and incubated for 1 h at 20°C with antibody to SGLT1 in PBS containing bovine serum albumin (1 mg/ml). After washing with PBS, the oocytes were incubated for 1 h at 20°C with Alexa Fluor 546-conjugated goat antibody to rabbit immunoglobulin G (A-11010, Molecular Probes) in PBS containing bovine serum albumin (1 mg/ml) and then washed an additional three times with PBS. They were finally mounted with FluoroGuard (Bio-Rad) and observed with a confocal microscope (Micro Radiance 2000, Bio-Rad).

3. Results

3.1. Characterization of SGLT1 mutations

We sequenced the 15 exons of SGLT1 and their flanking regions from the proband, her parents and sister, and two control subjects. With the proband’s DNA, five differences were detected between the sequences obtained and the original published SGLT1 sequence (GenBank accession numbers HUMSGLT01–12). The initial sequence analysis indicated the presence of two heterozygous mutations. To verify the presence of these mutations, we sequenced several clones of each exon. About half of the patient’s exon 5 clones exhibited a CGG→CTT transition at codon 135, which would result in an Arg^{135}→Trp mutation (Table 1); the remaining exon 5 clones of the proband showed the normal sequence. The patient’s father showed a similar pattern at exon 5 in that 11 clones showed the same CGG→CTT transition and the remaining seven clones exhibited the normal sequence. The mother and sister of the patient as well as the two normal control subjects showed exclusively the wild-type sequence for exon 5.

We detected another mutation, CAGtaggtatatct→CAgacct, in half of the clones derived from the boundary of exon 12 and intron 12 of the patient
Table 1
Number of cloned exons derived from SGLT1 of a GGM patient and her family

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Genomic DNA derived from the patient, her parents, and a sister was subjected to PCR with primers that result in the amplification of each of the 15 exons of SGLT1 (exon 15 was divided into proximal (p) and distal (d) regions). Individual clones corresponding to each PCR product were sequenced, and the numbers of normal and mutant clones are shown.

Fig. 1. Mutation in intron 12 of SGLT1 in the patient and her mother. (A) Genomic DNA isolated from the patient, her parents, and a control subject was subjected to PCR with a forward primer containing the normal (N) or mutant (M) intron 12 sequence and the reverse primer, 5'-CCATACCAAACCTCCGAGGAG. The PCR products were subjected to electrophoresis and stained with ethidium bromide. (B) Genomic DNA was subjected to PCR with primers that amplify DNA fragments containing the splice donor site for intron 12 of SGLT1 (forward, 5'-TACTCATGTAGAGCTATTTA; reverse, 5'-CCATACCAAACCTCCGAGGAG). The PCR products were treated with MvaI, which is predicted to generate 280-, 63-, 50-, and 26-bp fragments from the 419-bp normal sequence and 136-, 136-, 63-, 50-, and 26-bp fragments from the 411-bp mutant sequence. The band above the expected 280-bp fragment for the patient and her mother was shown by DNA sequencing to contain a mixture of the normal and mutant PCR products.
Her mother showed the same change in four of 10 clones, whereas all the corresponding clones derived from her father and sister were normal. To confirm the mutation in intron 12, we first performed PCR with a primer corresponding to the normal sequence (5'-AATGAGCCAGTAGTGCAT) or a primer based on the mutant sequence (5'-GAGAGTCAATGAGCCAGACC) (Fig. 1A). With DNA derived from the patient or her mother as template, PCR products were obtained with both the normal primer and the mutant primer, whereas DNA derived from the father or a control subject yielded a PCR product only with the normal primer. We also subjected PCR products spanning the intron 12 donor splice site to treatment with MvaI (Fig. 1B), given that the intron 12 mutation creates a new site for this enzyme. A pattern of digestion products consistent with the presence of the new MvaI site was apparent only with DNA from the patient and her mother (Fig. 1B).

The remaining three differences between the SGLT1 sequences found in all the sequences determined in the present study and the original published sequence have also been detected by others (GenBank accession numbers HUMSGLT1, HSCB1E7, HS65B7, and HS12724) and are as follows: exon 1, 5'-ATGGAGCAGTAGGCAT (HUMSGLT1) versus 5'-ATGGAGCAGTAGGCAT (HUMSGLT1, HSCB1E7, and four subjects in this study); intron 12, 5'-TTCAGAGATCCACTAGTTTCACTAG (HUMSGLT09) versus 5'-TTCAGAGATCCACTAGTTTCACTAG (HS65B7 and six subjects in this study); and intron 15, 5'-GCCGCAGAAGGAGATGTC (HUMSGLT12) versus 5'-GCCGCAGAAGGAGATGTC (HS12724 and three subjects in this study). These differences may correspond to polymorphisms or to errors in the original sequence.

3.2. Glucose transport activity and immunoblot analysis of SGLT1 mutants

To confirm that the SGLT1 mutations detected in the proband are responsible for her disease, we injected mRNA corresponding to the normal or mutant SGLT1 alleles into Xenopus oocytes and measured glucose transport activity. Because the effect of the intron 12 mutation on splicing is not known, we examined two possible forms of SGLT1 mRNA that either contain the intron 12 sequence (intron 12-read-through form) or lack the exon 12 sequence (exon 12-skipping form). The glucose transport activity of oocytes expressing either of the three SGLT1 mutants (Arg135Trp, intron 12-read-through, or exon 12-skipping) was <5% of that of those expressing the wild-type protein, and was therefore indistinguishable from that of water-injected oocytes (Fig. 2). Immunoblot analysis of injected oocytes revealed that the wild-type transporter migrated as a diffuse band with an apparent molecular size of 76 kDa (Fig. 3). In contrast, the Arg135Trp mutant migrated as two high-molecular-weight aggregates and a minor band corresponding to the position of the core-glycosylated transporter [16]. Since the intron 12-read-through mutant and the exon 12-skipping mutant are devoid of the antigenic site, no immunological analysis was performed on these mutants.
3.3 Immunocytochemical analysis of the Arg135→Trp mutant

The trafficking of SGLT1 in injected oocytes was examined by immunocytochemical analysis with a confocal microscope and antibody to the COOH-terminal of SGLT1. The pattern of specific staining indicated that both the wild-type protein and the Arg135→Trp mutant of SGLT1 were localized to the cell surface (Fig. 4). The Arg135→Trp mutant or wild-type SGLT1 that were fused with green fluorescent protein at the COOH-terminal was also localized to the cell surface of oocytes, although neither of these fusion proteins exhibited significant glucose transport activity (data not shown).

4. Discussion

More than 30 mutations of SGLT1 responsible for GGM have been identified by Wright and coworkers (http://archive.uwcm.ac.uk/uwcm/mg/search/120375.html). The patient described in the present study exhibited symptoms typical of GGM, and treatment with fructose-substituted formula proved effective. The Arg135→Trp mutation detected in one of the proband’s SGLT1 alleles was derived from her father, and the mutation in intron 12 of her other SGLT1 allele was inherited from her mother. Both of these mutations abolish the glucose transport activity of SGLT1, as shown by the expression of the corresponding proteins in Xenopus oocytes. Our results thus indicate that the patient is a compound heterozygote for mutations in SGLT1.

The mutation in intron 12 of SGLT1 identified in the proband has not previously been detected in other GGM patients. Although the final processed form of the transporter encoded by the mutant allele is not
known, we examined the activity of two possible such forms, an exon 12-skipping mutant and an intron 12-read-through mutant, in the *Xenopus* oocyte expression system. Our observation that neither of these mutant proteins exhibited glucose transport activity is consistent with previous data showing that the COOH-terminal region of SGLT1 is important for glucose recognition [17].

The primary defect of 21 missense mutants of SGLT1 studied by Wright et al. [1] was mistrafficking, with preliminary results indicating that only one missense mutant, Glu$^{457}$ → Arg, retained the ability to translocate to the plasma membrane of *Xenopus* oocytes. Although the Arg$^{135}$ → Trp mutant has been described previously [18], its properties were not studied. We have now shown that this mutant is expressed in *Xenopus* oocytes at a level similar to that of the wild-type protein, and that it undergoes apparently normal trafficking to the plasma membrane. However, the mutant protein failed to exhibit glucose transport activity and appeared to form aggregates. These results indicate that the Arg$^{135}$ → Trp mutant of SGLT1 is localized to the plasma membrane but is non-functional, probably as a result of an abnormal conformation. The roles of positively charged amino acid in the vicinity of loops connecting transmembrane segments have been studied by von Heijne and coworkers [19,20]. Thus, one possibility is that the Arg$^{135}$ → Trp mutation affects the translocation of the following transmembrane segment [19]. Another possibility is that Arg$^{135}$ exerts the ‘snorkel’ effect and contributes to the fine positioning of the transmembrane segment [20]. In any event, this mutant is a rare example of a non-functional SGLT1 protein and may be important for the molecular studies on the topology of membrane proteins as well as on the structural basis of sugar transport.

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