Functional role of specific amino acid residues in human thiamine transporter \textit{SLC19A2}: mutational analysis

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\textit{SLC19A2} is a membrane thiamine transporter expressed in a variety of human tissues, including the gastrointestinal tract. Little is currently known about the structure/function relationship of \textit{SLC19A2}. We examined the effect of introducing mutations in \textit{SLC19A2} identical to those found in thiamine-responsive megaloblastic anemia syndrome (TRMA), on functional activity and membrane expression of the transporter. We also examined the effect of mutating the only conserved anionic residue (E138) in the transmembrane (TM) domains of \textit{SLC19A2} and of the putative glycosylation sites (N63, N314). Northern blot analysis showed \textit{SLC19A2} mRNA was expressed at the same level in HeLa cells transfected with wild-type or mutated \textit{SLC19A2}. Introducing the clinically relevant mutations (D93H, S143F, G172D) led to a significant ($P < 0.01$) inhibition of thiamine uptake. Mutations of the two potential N-linked glycosylation sites (N63Q, N314Q) of \textit{SLC19A2} did not affect functional activity; they did, however, lead to a noticeable reduction in apparent molecular weight of protein. Western blot analysis showed all proteins (except D93H) were expressed in the membrane (not the cytoplasmic) fraction of HeLa cells. These results provide direct confirmation that clinically relevant mutations in \textit{SLC19A2} observed in TRMA cause malfunctioning of the transporter and/or a defect in its translation/stability. Results also show conserved TM anionic residue of the \textit{SLC19A2} protein is critical for its function. Furthermore, native \textit{SLC19A2} is glycosylated, but this is not important for its function.

thiamine transport; site-directed mutagenesis; membrane transport; thiamine-responsive megaloblastic anemia syndrome

WATER-SOLUBLE VITAMIN thiamine is involved as a cofactor in many critical cellular reactions, and its deficiency leads to a variety of clinical abnormalities including cardiovascular and neurological disorders (2, 26, 27). Human and mammalian cells cannot synthesize thiamine and thus must obtain the vitamin from exogenous sources via transport across the cell membrane. Previous physiological and biochemical studies have shown the process of cellular uptake of thiamine across the plasma membrane to be via a carrier-mediated mechanism(s). This includes transport of the vitamin in absorptive epithelial cells of the human small and large intestine (4, 8, 11, 19, 20). Recently several groups have identified a new human gene, \textit{SLC19A2}, that encodes a thiamine transporter, and cDNAs from human heart, skeletal muscle, fibroblast, and placenta have been reported (3, 5, 15). Mutations in this gene are believed to be the cause of thiamine-responsive megaloblastic anemia syndrome (TRMA) (3, 6, 10, 17, 21, 24). This human thiamine transporter is predicted to encode a multitransmembrane protein that, when expressed in mammalian cells, specifically induces thiamine uptake (5, 20). Recent studies in our laboratory have shown that this gene is also expressed in different tissues of the human gastrointestinal tract, raising the possibility that this transporter may play a role in the normal human intestinal thiamine absorption process (18). However, to date, there has been very little known about the structure-function relationship of \textit{SLC19A2}, including the link between the reported mutations in \textit{SLC19A2} gene in TRMA and the function of the protein in transporting thiamine. In this study, we used site-directed mutagenesis and examined the importance of specific amino acid residues in the \textit{SLC19A2} polypeptide on its function. Specifically, we examined the effect of introducing mutations in \textit{SLC19A2} identical to those found in TRMA patients on thiamine uptake. We extended our study further to understand the \textit{SLC19A2} functions on the basis of its putative secondary structure model (Fig. 1). In this context, we also examined the effect of mutating the only conserved anionic amino acid residue in any of the \textit{SLC19A2} transmembrane (TM) domains, or two putative glycosylation sites on the ability of the protein to transport the cationic thiamine. Mutants and wild-type \textit{SLC19A2} cDNA were expressed in HeLa cells, and the level of expression and thiamine uptake was determined and compared. Results showed that the clinically relevant mutations in \textit{SLC19A2} (D93H, S143F, and G172D) cause malfunctioning of the thia-
mine transporter and/or a defect in its translation/stability. Results also showed that the conserved anionic residue in the predicted fourth TM domain of the SLC19A2 protein is critical for its function. Furthermore, native SLC19A2 appears to be glycosylated, but this glycosylation does not appear to be important for its function.

MATERIALS AND METHODS

[3H]thiamine (specific activity 555 GBq/mmol; radiochemical purity >98%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). HeLa cells were purchased from American Type Tissue Culture Collection (Rockville, MD) and routinely cultured in MEM with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO2. TRIzol reagent and Lipofectamine 2000 were purchased from Life Technologies (Rockville, MD). Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad (Hercules, CA). DNA oligonucleotide primers were ordered from Sigma Genosys (Woodlands, TX). Routine biochemicals, enzymes, and cell culture reagents were all of molecular biology quality from Sigma (St. Louis, MO).

Functional Expression of the SLC19A2 in HeLa Cells

Vaccinia virus expression system. vTF7.3 vaccinia virus encoding T7RNA polymerase and pVOTE.1 plasmid vector were obtained through the generosity of Dr. Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Vaccinia is a complex DNA virus that replicates in the cytoplasm of most mammalian cells, encodes RNA polymerase and transcription factors, and accommodates large amounts of recombinant DNA (13, 14). Synthesis of T7 RNA polymerase by a recombinant vaccinia virus leads to a high level of expression of genes placed next to a T7 promoter within a transfected plasmid or a second coinfecting virus (28). In the present study, the full-length SLC19A2 cDNA was inserted into the pVOTE.1 expression vector in such a way that the sense transcription of the cDNA is under the control of the T7 promoter in the plasmid. This made the use of transient vaccinia virus expression system for functional characterization of the wild-type and mutant SLC19A2 gene possible.

Site-directed mutagenesis. Mutations in SLC19A2 were introduced by site-directed mutagenesis (9) using the SLC19A2 cDNA corresponding to the open reading frame (ORF) and cloned into the mammalian expression vector pDNA3.1. Mutations were made to introduce the clinically relevant missense mutations reported in patients with TRMA (D93H, S143F, and G172D; see Ref. 17), negative charge residues (E28A, E66A, E138A, and D444A), and the potential N-linked glycosylation sites (N63Q and N314Q).

Fig. 1. Schematic diagram of the putative secondary structure of human thiamine transporter protein. Positions of the 12 transmembrane-spanning segments are indicated by numbering [1–12]. Arrows indicate mutations made on the basis of thiamine-responsive megaloblastic anemia syndrome (TRMA) clinical reports [D93H (France), S143F (Brazil) and G172D (Italy)], (see Ref. 17), negative charge residues (E28A, E66A, E138A, and D444A), and the potential N-linked glycosylation sites (N63Q and N314Q).

Table 1. Internal primers used for generating mutations in human SLC19A2.

<table>
<thead>
<tr>
<th>Mutagenic Primers</th>
<th>N63Q 5’-CTGCTGGGCGGCAAGCAGGCTGACCGAGAG-3’</th>
<th>N314Q 5’-GTGGCTATTTTTTCAAGTTGTG-3’</th>
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<tbody>
<tr>
<td></td>
<td>E183A 5’-GGCATTGCCAAGACGCTGCAATGCCATTACTCTTATACTC-3’</td>
<td>E28A 5’-CCGCTCCCGGTCTGCGGCTTCTTCTTG-3’</td>
</tr>
<tr>
<td></td>
<td>D93H (France) 5’-GGGGAAGATCTGAGGGGTTTCCAGATTCCCTCTTAACT-3’</td>
<td>G172D (Italy) 5’-GTGGCCTATTACTTTATATACCTGTCGGAGCTGGCCG-3’</td>
</tr>
<tr>
<td></td>
<td>D444A 5’-GCTCACTCTCTATTGTGTGAGCTGCCAGTGGCTCTT-3’</td>
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Nucleotides that encompass the desired codon changes were used for site-directed mutagenesis.
transfection reagent (Life Technologies, Gaithersburg, MD) following the manufacturer’s procedure. The SLC19A2 in pcDNA 3.1/His contains the codons for a 6-histidine-tag as well as an epitope for an antibody. This allows easy identification and purification of the expressed protein. Cells were grown in flask culture, and the whole cell extracts were used to evaluate the level of SLC19A2 protein expression. The His-tagged proteins were purified from these extracts using a proband column (Invitrogen, Carlsbad, CA) and the manufacturer's instructions. Protein concentrations were determined using the Bio-Rad detection system on a Beckman DU640B spectrophotometer.

Western Blot Analysis

Immunoblotting was used to address the question of whether the His-tagged SLC19A2, and mutant proteins were inserted into the plasma membrane or not. For the purpose, the plasma membrane and cytoplasmic fractions were isolated from the whole cell extract of transfected HeLa cells following an established procedure (1, 25) and then were purified by proband column before applying them onto SDS-PAGE. Equal amounts of protein were loaded on 10% polyacrylamide gels (SDS-PAGE) and resolved at 200 V for 4 h in Tris-glycine SDS running buffer. Proteins were transferred onto a PVDF membrane at 35 mA for overnight at 4°C in wet transfer buffer. Membranes were blocked for 2 h at room temperature with PBS-Tween 20 (PBS-T) buffer containing 5% (wt/vol) nonfat dry milk powder. Membranes were then incubated overnight at 4°C with anti-Xpress antibody (1: 5,000 in PBS-T buffer), and washed four times with PBS-T buffer. The secondary antibody (anti-mouse IgG-peroxidase conjugate diluted in 1:5,000 in PBS-T buffer), and washed four times with PBS-T buffer. Proteins were detected using enhanced chemiluminescence substrate (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions, and exposed to Kodak X-ray film for periods of 30 s to 5 min. Blots were rechecked for the presence of SLC19A2 protein by using specific polyclonal antibodies raised against a synthetic peptide of SLC19A2, as previously described by us (20).

Northern Blotting

Total RNA was isolated from the wild-type and mutant SLC19A2 transfected HeLa cells using Trizol reagent as per the manufacturer's procedure. For Northern analysis, equal amounts of total RNA were loaded on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes by standard method. Blots were stained with ethidium bromide to estimate the quantitative loading variations. Hybridization was carried out as described previously (18). Full-length ORF SLC19A2 cDNA was randomly labeled with [32P]dCTP and used as a probe for detection. Final washing was performed in 0.2X SSC, 0.1% SDS for 15 min at 65°C. Membranes were exposed to Kodak X-ray films, and autoradiographs were analyzed. By the use of densitometry, data were normalized relative to human β-actin.

Thiamine Uptake Studies

Uptake studies were performed on confluent monolayers of HeLa cells incubated in Krebs-Ringer buffer (in mM): 133 NaCl, 4.93 KCl, 1.23 MgSO4, 0.85 CaCl2, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 7.4, at 37°C. [3H]thiamine (0.3 μCi/ml; 15.0 nM) was added to the incubation medium at the onset of the uptake experiment. Uptake was examined over a period of 3 min, i.e., initial rate (unpublished observations), and the reaction was terminated by the addition of 2 ml of ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer and digested with 1 ml of 1 N NaOH, neutralized with HCl, and then counted for radioactivity. Protein contents of cell digest were measured on parallel wells using a Bio-Rad kit (Richmond, Virginia).

Statistical Analysis

All measurements were made in triplicate and each experiment was repeated three times with separate transfections. Uptake results presented in this paper are means ± SE of multiple separate uptake determinations and were expressed by femtomoles per milligrams of protein per unit of time. Statistical differences were analyzed by Student’s t-test, with statistical significance set at 0.05 (P < 0.05) relative to simultaneously run controls.

RESULTS

Expression of SLC19A2 in HeLa Cells: Direct Evidence for Functional Impairment of SLC19A2 in TRMA

In this study, we first validated the ability of the vaccinia virus for use in the expression of SLC19A2 in HeLa cells by transient transfection. This was performed by examining the initial rate (3 min; data not shown) of thiamine (15.0 nM) uptake in HeLa cells transfected with SLC19A2 and those of controls, i.e., cells transfected with pVOTE.1 lacking the SLC19A2 insert and nontransfected cells. Results showed significant (P < 0.01) induction in thiamine uptake in SLC19A2 transfected cells compared with controls (Fig. 2).

In another study, we examined the effect of introducing identical missense mutations to those found in...
TRMA patients on transport function of SLC19A2.

TRMA is a rare autosomal recessive disease caused by mutations in the SLC19A2 gene (3, 6, 10, 17, 21, 24). Mutations were introduced as follows: D93H, S143F, and G172D. Mutant or wild-type SLC19A2 were then expressed in HeLa cells as described in MATERIALS AND METHODS. Results showed that all mutations lead to a significant (P < 0.01) inhibition in thiamine uptake compared with wild-type SLC19A2 (Fig. 2). We also examined, using Northern blot analysis, the level of mRNA expression of the different mutants in trans-fected HeLa cells. Results showed a similar level of mRNA expression of mutants and wild-type SLC19A2 (Fig. 3). Also, we determined whether the mutated SLC19A2 protein is expressed in HeLa cells or not, and whether the expression is at the cell membrane. Western blot analysis using either anti-Xpress monoclonal antibody directed against an epitope in pcDNA 3.1/His or a specific polyclonal antibodies directed against the SLC19A2 protein were employed. Results showed that the wild-type protein and two of the TRMA mutants (G172D and S142F) are expressed (~66 kDa) at a similar level in the cell membrane fraction of these cells (Fig. 4; lanes 3 and 4); no expression was found in the cytoplasmic fractions for either mutants. In contrast, the protein of the D93H mutant was found neither in the cell membrane fraction nor the cytoplasmic fraction of HeLa cells (Fig. 4; lanes 5 and 6).

**Role of Anionic Amino Acids in SLC19A2 Function**

Recent studies have shown that the transport of charged substrates is influenced by amino acid residues in the TM domain of their transporters that carry opposing charges (23). In this study, we examined the effect of mutating the only conserved anionic amino acid in any of the TM domains of SLC19A2 (E138A) on the ability of the carrier protein to transport the positively charged (cationic) thiamine molecule. We also mutated few anionic amino acid residues outside the TM domains of the SLC19A2 polypeptide for comparison (E28A, E66A, and D444A). Results showed mutating the only conserved anionic amino acid (E138A) lead to a significant (P < 0.01) inhibition in thiamine uptake (Fig. 2). In contrast, no inhibition in thiamine uptake was seen with the E28A, E66A, and D444A mutations (Fig. 2).

In another study, we determined the level of expression of the message of the E138A mutant in HeLa cells by Northern blot analysis and compared the results to that of wild-type SLC19A2 in HeLa cells (Fig. 3). Re-
SLC19A2 Glycosylation: Role in Function and Expression of the Thiamine Transporter at Cell Membrane

SLC19A2 has two potential N-glycosylation sites, one at N63 and the other at N314. It is not known, however, if either of these sites is actually glycosylated and if glycosylation affects function and/or membrane expression of the thiamine carrier protein. To investigate these issues, we mutated the potential N-glycosylation sites (N63Q, N314Q) simultaneously or sequentially and examined the effect of these mutations on thiamine uptake, expression of SLC19A2 at cell membrane, and mutant protein. Results showed that simultaneous or sequential mutations of the potential N-linked glycosylation sites of SLC19A2 have no significant functional consequences on thiamine uptake (Fig. 2). We also examined the effect of treating HeLa cells expressing the wild-type SLC19A2 with the glycosylation inhibitor tunicamycin (2 μM for 24 h) on thiamine uptake. Results showed that tunicamycin had no significant affect on thiamine uptake in the treated cells compared with control (Fig. 2).

In another study, we examined whether the N63Q/E314Q mutations affect membrane expression of the SLC19A2 protein. This was performed using Western blot analysis as described in MATERIALS AND METHODS. Results showed that the N63Q/N314Q mutant is expressed in the cell membrane fraction of HeLa cells (Fig. 4; lane 11) and that the level of expression is similar to that of the wild-type; no expression was found in the cytoplasm in either case. However, the predominant band evident in the wild-type SLC19A2 transfected cells at ~66 kDa (Fig. 4; lane 10) was shifted to ~56 kDa after mutation (Fig. 4; lane 11). A similar reduction in the apparent molecular weight of the wild-type SLC19A2 protein was also observed in transfected cells treated with tunicamycin (Fig. 4; lane 12). However, cells transfected with the mutated potentially N-linked glycosylation sites individually show the 66 kDa-band to be either smeared or only slightly shifted from its original position (Fig. 4; lanes 9 and 10).

DISCUSSION

The aim of this study was to gain some insight into the structure-function relationship of the recently cloned thiamine transporter SLC19A2. Specifically, we sought to provide direct evidence for a functional defect in SLC19A2-caused mutations found in TRMA patients, and to determine whether this is the result of a lack of expression of the protein at the cell membrane. We also sought to examine the effect of mutating the only conserved anionic amino acid residue in any of the SLC19A2 TM domains and that of the two putative glycosylation sites on the ability of the protein to transport the cationic thiamine and expression of the protein at the cell membrane. All cDNAs were then expressed in HeLa cells, and the level of their expression and thiamine uptake was determined and compared.

TRMA is an autosomal recessive disorder with features that include megaloblastic anemia, diabetes mellitus and sensory-neural deafness. Missense and nonsense mutations in SLC19A2 have both been reported in patients with this disorder. In this study, we focused only on the missense mutations identified in three different families from three different geographic locations. Mutations included a D93H mutation, a negatively charged residue (aspartic acid) that was mutated into a positively charged residue (histidine); a S143F mutation, a hydrophilic amino acid (serine) that was mutated to a hydrophobic amino acid (phenylalanine); and a G172D mutation, a nonpolar hydrophilic amino acid (glycine) that was mutated to a negatively charged hydrophilic amino acid (aspartic acid). All these mutations were located in exon two of the SLC19A2 gene. Our results showed that all mutants caused significant impairment in thiamine uptake following expression in HeLa cells. This impairment in thiamine uptake was not due to a difference in the level of expression of the SLC19A2 message, because similar levels of mRNA were found (by Northern blot analysis) for these mutants and the wild-type SLC19A2. However, protein levels of the mutants were found to vary as shown by the Western blot analysis. Mutants S142F and G172D were both found to be expressed in the cell membrane fraction of HeLa cells and at a comparable level to that of wild-type protein. In both cases, no expression of the protein was detected in the cytoplasmic fraction of the transfected HeLa cells. In contrast, mutant D93H was detected neither in the membrane nor in the cytoplasmic fractions of HeLa cells, indicating that either the transfected cells were unable to translate this mutant or there is a defect in the stability of this mutant protein. Findings that both S142F and G172D mutants were expressed in the cell membrane (not cytoplasmic) fraction of transfected cells suggest that the defect in thiamine uptake in these particular TRMA mutations is not the result of impaired trafficking of the SLC19A2 protein to the cell membrane; rather the defect appears to be in the function due to impairment of the thiamine carrier. These findings are interesting compared with the mutations found in the glucose transporter SGLT1 in patients with glucose-galactose malabsorption, another autosomal recessive disorder. In this disorder, many of the mutations lead to a defect in intracellular trafficking of the protein to the cell membrane (12). Recent studies (23) have shown transport of charged substrates to be influenced by amino acid residues in the TM domain of their transporters that carry an opposing charge. SLC19A2 protein has only one con-
served anionic amino acid residue in any of its TM domains located in the fourth TM stretch. The effect of mutating this amino acid residue was found to lead to a significant inhibition in uptake of the cationic thiamine. This inhibition was not due to a decrease in the level of message and protein expression of the mutated protein as shown by the results of the Northern and Western blot analysis, respectively. Rather, the effect appears to be mediated via impairment in the function of the thiamine carrier itself. This, raises the possibility that this amino acid residue may play a role in the transport function of the thiamine carrier. It is interesting to mention here that mutating anionic residues outside the TM domains of SLC19A2 (E28A, E66A, and D444A) did not affect the function of the thiamine transporter, thus providing support to this suggestion. Further studies are required to establish this possibility.

Glycosylation of proteins has been reported to play a role in folding and membrane targeting of that protein (7, 22). SLC19A2 protein has two potential glycosylation sites (5, 6) but nothing is known about their glycosylation status. Therefore, we examined the effect of simultaneous and sequential mutation of these sites on transport function and membrane expression of SLC19A2. Our findings showed that alteration of these sites cause a significant shift in the SLC19A2 protein from ~66 to ~56 kDa. This clearly indicates that the native SLC19A2 protein is glycosylated in living cells. Mutating the glycosylation sites, however, did not affect thiamine uptake. Similarly, such mutations did not affect the expression of the SLC19A2 protein at the cell membrane. The latter observation suggests that glycosylation of SLC19A2 is not important for intracellular trafficking/targeting of the protein to cell membrane. These findings were confirmed by the studies with the glycosylation inhibitor tunicamycin. In these studies, treatment of HeLa cells expressing the wild-type SLC19A2 with this inhibitor did not affect the transport function of the protein. It did, however, lead to a significant reduction in the apparent molecular weight of the protein to a level similar to that seen with the mutation approach.

In summary, our findings provide direct confirmation that the clinically relevant mutations in SLC19A2 in TRMA patients cause malfunctioning of the transporter per se or a defect in its translation/stability. Results also show that the conserved anionic residue in the predicted fourth TM domain of the SLC19A2 protein is critical for its function. Furthermore, native SLC19A2 appears to be glycosylated, but this glycosylation may not be important for its function.

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