Cloning of the Human Thiamine Transporter, a Member of the Folate Transporter Family*

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We have isolated a cDNA from human placenta, which, when expressed heterologously in mammalian cells, mediates the transport of the water-soluble vitamin thiamine. The cDNA codes for a protein of 497 amino acids containing 12 putative transmembrane domains. Northern blot analysis indicates that this transporter is widely expressed in human tissues. When expressed in HeLa cells, the cDNA induces the transport of thiamine ($K_t = 2.5 \pm 0.6 \mu M$) in a Na$^+$-independent manner. The cDNA-mediated transport of thiamine is stimulated by an outwardly directed H$^+$ gradient. Substrate specificity assays indicate that the transporter is specific to thiamine. Even though thiamine is an organic cation, the cDNA-induced thiamine transport is not inhibited by other organic cations. Similarly, thiamine is not a substrate for the known members of mammalian organic cation transporter family. The thiamine transporter gene, located on human chromosome 1q24, consists of 6 exons and is most likely the gene defective in thalassemic anemia. At the level of amino acid sequence, the thiamine transporter is most closely related to the reduced-folate transporter and thus represents the second member of the folate transporter family.

Thiamine, as a component of the coenzyme thiamine pyrophosphate, is important for glycolysis and energy production in mitochondria (1). It is an essential nutrient, and its requirement in the developing fetus is met by the transplacental transport from maternal circulation to fetal circulation. The concentration of thiamine is more than 10-fold higher in fetal umbilical vein plasma compared with that present in maternal plasma (2), suggesting the presence of an efficient transport system for the vitamin in placenta. The underlying mechanism of transplacental transport of thiamine has been investigated using in vitro perfusion techniques (3, 4) as well as isolated placental brush border membrane vesicles (5). Thiamine transport has also been functionally characterized in other tissues/cells such as intestine (6–9), erythrocytes (10), hepatocytes (11), and neuroblastoma cells (12). These studies have shown that the transport of thiamine is a carrier-mediated, energy-dependent process.

Thiamine is an organic cation. Depending on the pH of the solution, thiamine can exist either as a monovalent or bivalent cation (6). Absorbptive tissues such as placenta (13, 14), kidney (15, 16), and intestine (17) are known to express specific organic cation/H$^+$ antiport systems that are capable of transporting both endogenous as well as exogenous organic cations. The presence of an outwardly directed proton gradient was shown to induce concentrative thiamine accumulation in brush border membrane vesicles isolated from human term placenta, suggesting a possible role of an organic cation/H$^+$ antiport system in the uptake of thiamine into the placenta (5). Consistent with such an uptake mechanism, the thiamine uptake into placental brush border membrane vesicles was abolished in the presence of proton ionophores and unaffected by the presence of an inside-negative membrane potential. Existence of a similar uptake mechanism for thiamine has also been described in intestine (8) and erythrocytes (10).

In this study, we describe the molecular cloning of a cDNA from human placenta which, when expressed in mammalian cells, induces the uptake of thiamine. This thiamine transporter (ThT1)§ shares significant homology (40% identity) with the reduced-folate transporter (RFC or FOLT) previously cloned from human placenta (18). Northern analysis indicates that the ThT1-specific transcript (~3.8 kb in size) is widely expressed in human tissues. The human gene of ThT1 (~22 kb), which has already been sequenced as a part of the Human Genome Project, maps to chromosome 1q24 and consists of 6 exons and 5 introns as determined by comparison of sequences of tht1 gene and ThT1 cDNA.

EXPERIMENTAL PROCEDURES

Materials—SuperScript Plasmid System for cDNA cloning, Dulbecco’s modified Eagle’s medium, and Lipofectin were purchased from Life Technologies, Inc. Nitropure transfer membranes were obtained from Osmonics (Minnetonka, MN). [3H]Thiamine (specific activity, 400 mCi/μmol), [3’,5’,7,9-3H]5-methyltetrahydrofolic acid (specific activity, 30 Ci/μmol), [3H(G)]riboflavin (specific activity, 30 Ci/μmol), and [3,5,7,9-3H]methotrexate (specific activity, 19.7 Ci/μmol) were purchased from Moravek Biochemicals (Brea, CA); [3’,5’,7,9-3H]Folic acid (specific activity, 30 Ci/μmol), [methyl-3H]choline chloride (specific activity, 85 Ci/μmol), [l-]3-[(1-14C) tetraethylammonium (TEA) bromide (specific activity, 55mCi/μmol), and [methylation-3H]MPP (specific activity, 60 Ci/μmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [l-]Carboxyl-[14C]Ascorbic acid (specific activity, 16.6 mCi/μmol), [N-methyl-3H]cimetidine (specific activity, 18.2 Ci/μmol), [α-32P]dCTP (specific activity, 3000Ci/μmol), and the Ready-to-Go oligolabeling kit were procured from Amersham Pharmacia Biotech. The cDNA for vOct2 (19, 20) was kindly provided by Dr. J. B. Pritchard (NIEHS, National Institutes of Health, Raleigh, NC). The original re-
ports on the cloning of rOCT3 (21) and rOCT2 (22) from rat placenta were from our laboratory. The cDNA for rOCT1 (23) was obtained by screening a rat liver cDNA library using rOCT3 as the probe. The cDNA for rOCTN1 was obtained by screening a rat placental cDNA library using rOCT1 as the probe (24).

**cDNA Cloning and Sequencing—**Previous studies from our laboratory have led to the cloning of the RFC from human placenta (18). Blast search of the GenBank peptide sequence data base using the amino acid sequence of human RFC showed that a sequence entry of a RFC-like protein has been submitted to the data base (accession CAA15926). This protein, as per the GenBank entry, is 468 amino acids long, truncated at the C terminus lacking the initiator methionine and shared 39% identity and 55% similarity with human RFC. The amino acid sequence of the RFC-like protein was derived by GENSCAN prediction from the nucleotide sequence of a genomic clone isolated from a genomic library of human chromosome 1 (nucleotide sequence data base accession no. AL021068, submitted by C. Bird, Sanger Center Chromosome 1 Mapping Group). Neither the complete amino acid sequence of the protein nor its function was known. In order to get the full-length cDNA of the RFC-like protein, we generated a cDNA probe specific to the protein by RT-PCR and screened a human placental cDNA library. The RT-PCR was done using poly(A)+ RNA isolated from human term placenta and the primers 5'–GCCAAGAGAGTCACAGTT-3' (upstream primer) and 5'–CAGGAGAAGAGAGAGATA-3' (downstream primer). The upper primer had an additional three nucleotides (underlined) added at the 5' end to ensure primer pair compatibility. The expected size of the RT-PCR product was 656 bp, comprising the region coding for Gln126 to Leu435 of the RFC-like protein. The identity of the product obtained was confirmed by sequencing before using it as the probe in library screening.

The screening of the placental cDNA library was done by colony screening of the plasmid cDNA library grown on Nitropure transfer membranes as described previously (25). The cDNA probe was labeled with [α-32P]dCTP by random priming using the Ready-to-Go oligolabeling kit. Positive clones were identified and the colonies purified by secondary screening. The size of the cDNA inserts of the positive clones was determined by restriction digestion using EcoRI/BamHI to release the insert from the vector followed by size fractionation by agarose gel electrophoresis. A single clone with the largest insert size was selected for further characterization.

Both sense and antisense strands of the cDNA were sequenced by primer walking. Sequencing by the dideoxynucleotide chain termination chemistry was performed by Taq DyeDeoxy terminator cycle sequencing using an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the BCM Search Launcher server (26) and NCBI server.

**Northern Analysis—**Tissue distribution of the ThT1-specific transcript was determined by Northern analysis. A hybridization-reagent, commercially available human multiple tissue blot (CLONTECH). The blot was probed sequentially with ThT1 cDNA and then with glyceraldehyde-3-phosphate dehydrogenase cDNA under high stringency conditions.

**Functional Expression of the ThT1 cDNA—**The cloned cDNA was functionally expressed in HeLa cells by vaccinia virus expression system as described previously (25, 27). Subconfluent HeLa cells grown on 24-well plates were first infected with the recombinant (VTF3a) vaccinia virus encoding T7 RNA polymerase and then transfected with 1 μg of the plasmid carrying the full-length cDNA using Lipofectin. In most experiments, transport measurements were made at 37 °C with a 10-min incubation using [3H]thiamine in the absence of Na+ to release 100% of the transport buffer was replaced with NaCl. When the influence of pH on transport was investigated, transport buffers of different pH were prepared by varying the concentration of Tris, Heps, and Mes. Transport was terminated by aspiration of the uptake buffer followed by two rapid washes with 2 ml of ice-cold transport buffer. The cells were then solubilized with 0.5 ml of 1% SDS in 0.2 N NaOH and transferred to vials for quantitation of the radioactivity associated with the cells. Endogenous transport was always measured in parallel in cells transfected with empty vector (pSPORT) and subtracted from the corresponding transport values measured in cells transfected with vector cDNA to obtain the cDNA-specific uptake of the vitamin.

**Data Analysis—**Uptake measurements were made either in duplicate or triplicate, and each experiment was repeated two to three times with separate transfections. Results are given as means ± S.E. of these replicate values. Kinetic analyses were carried out by nonlinear as well as linear regression methods using the commercially available computer program SigmaPlot (SPSS Inc., Chicago, IL).

**RESULTS AND DISCUSSION**

The cloned cDNA (ThT1, for thiamine transporter; GenBank accession no. AF160812) is 3530 bp long and has an open reading frame of 1494 bp including the termination codon. The open reading frame is flanked by a 96-bp-long 5′-noncoding sequence and a 1941-bp-long 3′-noncoding sequence. The open reading frame encodes a 497-amino acid protein (Fig. 1A) with a molecular mass of 55.4 kDa and a pI of 6.35. Hydrophathy analysis of the primary amino acid sequence using the Kyte-Doolittle method (28) with a window size of 17–24 amino acids per transmembrane helix predicts a topographical model with 12 putative transmembrane domains (Fig. 1B). The strongly preferred model for the arrangement of the transmembrane domains across the membrane is the one with both the N terminus and the C terminus toward the inside. There are two potential sites for N-linked glycosylation at positions 63 and 314 in putative intracellular domains. The primary amino acid sequence of ThT1 displays three sites (Ser8, Thr22, and Ser291) with consensus sequence for protein kinase C-dependent phosphorylation in putative intracellular domains. Interestingly, a 17-amino acid residue sequence, which is a signature of G-protein-coupled receptors (29), is also present in the amino acid sequence of ThT1 (amino acid residues 407–423).

A comparison of the amino acid sequence of human ThT1
with the protein sequences in the SwissProt sequence data base confirmed that ThT1 is identical to RFC-like protein, however, with an additional 29 amino acids at the N terminus. The closest relative of ThT1 is RFC1 (18), the reduced-folate transporter, with a sequence identity of 40% and similarity of 55% at the amino acid level. A comparison of amino acid sequence of ThT1 and RFC1 is presented in Fig. 1A. Thus, ThT1 is the second member of the folate transporter family to be cloned.

The expression of ThT1 in human tissues was investigated by Northern analysis using a commercially available multiple tissue blot containing size-fractionated poly(A)⁺ RNA obtained from several tissues of human origin. The ThT1-specific hybridization signal (3.8 kb in size) was widely distributed in human tissues (Fig. 2). The intensity of the hybridization signal obtained was especially high in skeletal muscle, followed by placenta, heart, liver, and kidney.

The functional expression of the clone was done in HeLa cells by transient transfection followed by vaccinia virus-induced expression of the cDNA. Since the cloned transporter showed significant homology to RFC1, we first investigated the ability of the cloned transporter to transport folate and its analogs. The uptake of [³H]5-methyltetrahydrofolate, [³H]folic acid, and [³H]methotrexate was studied in cells transfected with either empty vector or ThT1 cDNA in the presence (Fig. 3A) or absence (Fig. 3B) of Na⁺. These studies showed that none of these compounds was a transportable substrate for ThT1. We then tested the ability of ThT1 to transport several water-soluble vitamins like riboflavin, ascorbic acid, and thiamine. Of these substrates tested, only the uptake of thiamine was 2-fold higher in ThT1 cDNA-transfected cells in comparison to vector-transfected cells (Fig. 3). Removal of Na⁺ from the transport buffer did not abolish the transport activity, demonstrating that the transport activity mediated by ThT1 is Na⁺-independent. Subsequent experiments involving the characterization of the transport function of ThT1 were done in the absence of Na⁺.

The transport of thiamine in placental and intestinal brush border membrane vesicles was stimulated by an inside-out proton gradient (5, 8). We therefore examined the effect of extracellular pH on thiamine transport in vector- and cDNA-transfected cells (Fig. 4A). The uptake of thiamine at pH 8.0 was almost 3-fold higher than the uptake at pH 6.0. The process of thiamine uptake displayed a distinct pH optimum at about pH 8.0. Increasing the pH from 6.0 to 8.0 stimulated thiamine uptake, but further increase in pH above 8.0 inhibited the uptake. The endogenous thiamine transport measured in empty vector-transfected cells was much less compared with uptake in cells transfected with ThT1, and this endogenous uptake was similarly influenced by pH. The pH optimum of the endogenous transport, however, was not 8.0, and the uptake kept increasing even when the pH was increased to 8.5.

Thiamine is an organic cation. Recently, a number of organic cation transporters have been cloned and functionally characterized (30). The members of the organic cation transporter family accept a variety of organic cations as substrates. It was therefore of interest to see if any of these organic cation transporters is capable of transporting thiamine. We expressed all of the known members of the organic cation transporter family (OCT1, OCT2, OCT3, OCTN1, and OCTN2) in HeLa cells and compared the ability of these transporters to mediate the transport of thiamine and TEA, a prototypical organic cation substrate for these transporters. Although all of the organic cation transporters were functionally expressed, as seen by the increased TEA uptake in cDNA-transfected cells compared with vector-transfected cells, none of them, except OCT3, showed significant transport of thiamine (Table I).

The substrate specificity of ThT1 was evaluated by directly measuring the uptake of structurally diverse organic cations as well as by assessing the ability of these organic cations to inhibit the ThT1-mediated [³H]thiamine uptake. First, the uptake of radiolabeled choline, TEA, MPP, cimetidine, and thiamine was compared in cells expressing ThT1 and control cells...
transported cDNA Thiamine transport

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<th>cDNA specific (% control)</th>
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TABLE II

Substrate specificity of ThT1

HeLa cells were transfected with either pSPORT alone or ThT1 cDNA. Transfected cells were incubated with 5 μM [3H]thiamine for 10 min at 37 °C in the absence or presence of various unlabeled organic cations. After incubation, the cells were washed with ice-cold transport buffer, and radioactivity associated with the cells was quantitated. Final concentration of the unlabeled organic cations was 1 mM. Values in parentheses are percentage of corresponding control transport.

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Fig. 4. A, influence of pH on ThT1-induced thiamine transport in HeLa cells. HeLa cells were transfected with either pSPORT vector alone or pSPORT-ThT1 cDNA. Transport of [3H]thiamine (2.5 μM) in these cells was measured for 10 min at 37 °C using NMDG chloride-containing transport buffers of different pH. Values in parentheses represent percentage of the corresponding control uptake measured in cells transfected with empty vector. All these compounds are organic cations and are transported via organic cation/H+ antiport process in several tissues. However, there was no difference in the transport of TEA, choline, MPP, and cimetidine between control cells and ThT1-expressing cells (data not shown). Under identical conditions, the transport of thiamine was higher in ThT1-expressing cells than in control cells as expected. Next, the ability of various organic cations (1 mM) to inhibit the ThT1-mediated thiamine transport was investigated. Only unlabeled thiamine (1 mM) was able to inhibit the transport of [3H]thiamine (Table II). Other organic cations (guanidine, cimetidine, and choline) failed to inhibit thiamine uptake. These results provide confirmatory evidence that, unlike other organic cation transporters, ThT1 is very specific for thiamine. The transport process mediated by ThT1 was saturable with a K_{max} of 2.5 ± 0.6 μM for thiamine (Fig. 4B).

Comparison of the nucleotide sequence of ThT1 cDNA with the nucleotide sequence from which the amino acid sequence of RFC-like protein was derived indicated that the human gene coding for ThT1 has been sequenced in its entirety as a part of the Human Genome Project. The gene (~22 kb) maps to chromosome 1q24. A comparison of the nucleotide sequences of the tht1 gene and ThT1 cDNA has enabled us to deduce the exon-intron organization of the gene (Fig. 5). The gene consists of 6 exons and 5 introns. All exon-intron boundaries conform to consensus donor-acceptor sequences (gt/ag) for RNA splicing.

An autosomal recessive disorder called thiamine-responsive megaloblastic anaemia has been described (31), and the defect in this syndrome has been localized to chromosome 1, band 1q23.3 (32). Fibroblasts from these patients lack the high affinity transport system for thiamine. The ability of these cells to transport thiamine can be restored by transfecting the cells with the yeast thiamine transporter gene, THI10 (33). These findings suggest that, the primary abnormality in thiamine-
responsive megaloblastic anemia is the absence of a functional high affinity thiamine transporter and that the tht1 gene is most likely defective in these patients. Although both ThT1 and THI10 are thiamine transporters and THI10 is able to functionally correct thiamine transport defect, a comparison of the amino acid sequences of the two proteins by Blast search showed no significant homology between the two proteins.

In summary, we have cloned a human thiamine transporter from the placenta. Its transport function is Na⁺-independent. Structurally this transporter is related to the reduced-folate transporter whose transport function is also Na⁺-independent. Thus, ThT1 represents the second member of the folate transporter gene family. It is to be noted, however, that the substrate for ThT1 is an organic cation whereas the substrate for RFC1 is an organic anion. Even though both transporters are pH-dependent, the function of ThT1 is stimulated by an outward-directed H⁺ gradient, whereas the function of RFC1 is known to be stimulated by an inward-directed H⁺ gradient (34). The transport mechanism of these two transporters with respect to the involvement of H⁺ appears to be different. Transporters for other water-soluble vitamins have been previously identified and functionally characterized. The multivitamin transporter SMVT is specific for biotin and pantothenate and its transport function is obligatorily dependent on Na⁺ (25, 35, 36). Two different transporters (SVCT1 and SVCT2) have been identified for the transport of ascorbic acid (vitamin C) in mammalian cells, and both of them are obligatorily dependent on Na⁺ for their function (37). Thus, in addition to the differences in substrate selectivity, the vitamin transporters also appear to differ in Na⁺ dependence.

REFERENCES

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