Cloning and Functional Expression of a cDNA Encoding a Mammalian Sodium-dependent Vitamin Transporter Mediating the Uptake of Pantothenate, Biotin, and Lipoate*

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Vitamins are required for essential metabolic processes in all mammalian cells. Such cells have developed intrinsic mechanisms for active accumulation of essential vitamins. The small intestine and kidney, tissues that are important for the absorption of vitamins, possess active transport mechanisms that mediate the transcellular transfer of these essential nutrients. The placenta also functions in the transcellular transfer of nutrients from mother to developing fetus. The syncytiotrophoblast, the absorptive epithelium of the placenta, possesses specific transport systems for several nutrients (1). Recent studies (2–4) have demonstrated the presence of a Na⁺-dependent vitamin transporter in the placenta that accepts as substrates the water-soluble vitamins pantothenate and biotin and the essential metabolite lipoate. The active transport system responsible for the uptake of these compounds is driven by a transmembrane Na⁺ gradient. The uptake of [14C]pantothenate into human placental brush border membrane vesicles (4) and JAR human placental chorioncarnaoma cells (2) is mediated by a single saturable high affinity transport system with a Michaelis-Menten constant of 2–8 μM. The uptake of the radiolabeled pantothenate is inhibited by biotin and lipoate. The uptake of [3H]biotin into human placental brush border membrane vesicles is Na⁺-dependent and is inhibited by pantothenate and lipoate (3). Thus, a single transport system is responsible for the placental uptake of pantothenate, biotin, and lipoate. There is also evidence that a similar transport process for these vitamins operates in the small intestine and kidney in the absorption of these three essential vitamins (5, 6).

In this paper, we report on the cloning and functional expression of a rat placental cDNA that encodes a Na⁺-dependent vitamin transporter responsible for the placental uptake of pantothenate, biotin, and lipoate. This represents the first successful cloning of a Na⁺-dependent vitamin transporter from a mammalian tissue.

EXPERIMENTAL PROCEDURES

Materials—SuperScript Plasmid System for cDNA cloning and Lipofectin were purchased from Life Technologies, Inc. Restriction enzymes were obtained from Promega. Magna nylon transfer membranes were purchased from Micron Separations, Inc. d-[14C]Pantothenate (51.5 mCi/mmol) and [3H]biotin (58.2 Ci/mmol) were procured from NEN Life Science Products. The rat chromaffin granule amine transporter (rVMAT II) clone used in the screening was kindly provided by Dr. B. J. Hoffman (NIMH, Bethesda, MD). D-Pantothenate was purchased from ICN Biochemicals, Inc., and biotin, niacinamide, and α-tocopherol were bought from Sigma. HeLa cell line was obtained from ATCC (Rockville, MD) and routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml of penicillin, and 100 units/ml of streptomycin.

Construction of cDNA Library from Rat Placental mRNA—Rat placental mRNA was reverse transcribed using the SuperScript Plasmid System with a modified oligo(dT) primer adapter containing a NotI site. Following second strand synthesis and addition of SalI adapters, the cDNA was digested with NotI to generate cDNA with NotI and SalI “sticky” ends for unidirectional cloning. The cDNA was then size-fractionated by gel filtration in a Sephacryl column. cDNA larger than 1 kb1 was ligated to NotI-SalI-digested pSPORT vector and transformed into the competent DH10B strain of Escherichia coli by electroporation.

Screening of the cDNA Library—This was done by colony screening of the plasmid cDNA library grown on Magna nylon transfer membranes as described by Vogeli and Kaytes (7). The cDNA probe used for screening

1 The abbreviations used are: kb, kilobase(s); kbp, kilobase pair(s); bp, base pair(s); rCGAT, rat chromaffin granule amine transporter; SMVT, sodium-dependent multivitamin transporter; rSMVT, rat SMVT.

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A 1 MTVASTAAPS YTTSDTNRII STPSVDDYV YGGLVLLSLV IGLYHACRGW
101 GRHTVGELL ADRKMCCLTV ALSLLATPOS AVAILGQPAE YYPFTQYFWF
151 LGCSYFLGLL IPAHIFIPVFV YRLHTSAYE YLELRPKAV RIGGGTTPIF
201 QMVVMVQAL YAPSIALNAV TGDWMELSLVL ALGIVCNYIY ALGGLKAVIW
251 TDFQGTLME LGQLVYIVG AAAYUGGLHV WAVASQGHLI SGVLDPPDF
301 VRHTFVTATF GGVCMLSLY GVQQAQVQRY LSHSEKAAV LSCYAVEPCC
351 QVALCMSCLL GLVMFAYKT YSMSPQQBQA APEQLVLYFV MDLKKDMGPL
401 PGLPVACLPS GSLSTTSSAF NSLATVTDN LQIPWPPQLT HTRAIMLRS
451 LAFAYGLVCL GMAYVSSHLG SVLQAALSIF GMYGGPLGLG PGLNNFPFGCA
501 NPLGATVQGL TGTMAPWGQ IGSIVSRMSS AASAPPLNGS SSFLPNLTV
551 ATVTTLMPST LKPEFGQPF YSLSYLWYA HNSSTVIAVG LIVSLLTQGM
601 RKGRSLNPHTI YFPVFKLLAL LPLSCQKRLC WRSNHQDIIVT VTLNLPEKMG

FIG. 1. A, predicted amino acid sequence of rSMVT. Putative transmembrane domains are underlined. Sites for N-linked glycosylation (Ψ) are indicated. B, hydropathy plot of rSMVT using the Kyte-Doolittle method with a window size of 20 amino acids.

Functional Expression of the cDNA—The cDNA was functionally expressed in HeLa cells by vaccinia virus expression system (10) as described previously (11, 12). The cDNA was cloned in the plasmid pSPORT in such an orientation that the sense transcription of the cDNA was under the control of T7 promoter in the plasmid. Subconfluent HeLa cells grown in 24-well cell culture plates were first infected with a recombinant vaccinia virus (VTF7-3) encoding T7 RNA polymerase. This was followed by Lipofectin-mediated transfection of the plasmid cDNA into the cells. After 12 h post-transfection, transport measurements were made at room temperature using [14C]pantothenate and [3H]biotin. The transport buffer was composed of 25 mM Hepes/Tris (pH 7.5), supplemented with 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, and 5 mM glucose. The incubation time for transport measurements was 30 min. Transport was terminated by aspiration of the uptake buffer followed by two washes with 2 ml of ice-cold transport buffer. Following this, the cells were 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. The Na+ dependence of the transport process was examined by isoosmotically substituting NaCl in the transport buffer with choline chloride. HeLa cells transfected with empty...
RESULTS AND DISCUSSION

Screening of the Placental cDNA Library—Previous studies from our laboratory have provided evidence for the expression of an organic cation/H⁺ antiporter in the placenta (13, 14). With an intent to clone this transporter from the rat placenta, we screened a rat placental cDNA library with rCGAT as the probe. rCGAT transports monoamines, which are cationic at physiological pH, in exchange for protons. Thus there is a functional similarity between the organic cation/H⁺ antiporter and rCGAT. We therefore thought that the two proteins may have significant sequence homology and that the rCGAT cDNA probe may lead to the identification of the organic cation/H⁺ antiporter cDNA clone by cross-hybridization. When the rat placental cDNA library was screened under low stringency conditions with the rCGAT cDNA probe, several positive clones were identified. Plasmid cDNA was isolated from these clones, and the cloned inserts were released by restriction digestion with XbaI-SalI enzymes. The sizes of the inserts were analyzed on agarose gels, and clones larger than 2.0 kbp in size were sequenced at the 5’ end. Based on the nucleotide sequence so obtained, one clone was identical to the cloned rCGAT, another turned out to be rVMAT 1 (rSVAT) (8, 15), and several other clones did not have any homology to known cloned transporters. One of these clones, approximately 2.0 kbp in size, showed significant homology with known members of Na⁺-dependent glucose transporter family (Na⁺-dependent glucose transporters, Na⁺-dependent iodide transporter, Na⁺-dependent nucleoside transporters, and Na⁺-dependent myo-inositol transporter). This clone was partial and lacked the 5’ end of the cDNA including the translation start site. Therefore, a second screening of the same placental cDNA library was done using this partial cDNA as the probe. Three clones larger than 3.0 kbp were obtained, two of which turned out to be identical to the partial clone used as the probe except that they contained a ~1.0-kbp-long 5’ extension. One of these was arbitrarily chosen for further characterization by complete sequence analysis and functional expression.

Structural Features of the cDNA—The cloned cDNA is 3091 bp long and has an open reading frame of 1905 bp including the termination codon. The open reading frame is flanked by a 412-bp-long 5’ noncoding sequence and 774-bp-long 3’ noncoding sequence. The putative initiation codon is preceded by a Kozak consensus sequence (GTG AGG) (16). At the 3’ end, the cDNA has a polyadenylation signal (AATAAA) followed by a putative poly(A)⁺ tail. The open reading frame encodes a protein of 634 amino acids. The primary structure of this protein is shown in Fig. 1. The monomer has an estimated core molecular mass of 68.6 kDa and a pI of 7.86. Hydropathy analysis of the primary amino acid sequence using the Kyte-Doolittle method (17) with a window size of 20 amino acids shows that the protein is highly hydrophobic with 12 putative transmembrane domains (Fig. 1). When modeled as most other cloned transporters, both the N-terminal and C-terminal ends of the protein face the cytoplasm. Three putative N-glycosylation sites are present in the extracellular loop between transmembrane domains 11 and 12. The C-terminal end of the protein following the last transmembrane domain is about 84 amino acids long and is highly hydrophilic.

Functional Characterization—The functional expression of the clone was done in HeLa cells by transient transfection followed by vaccinia virus-induced expression of the cDNA. The function was monitored by the transport of radioactive substrates. Cells transfected with the empty vector were used to measure the endogenous transport activity. The ability of the expressed transporter to transport several substrates known to be transported by the cloned transporter was determined (Table 1). Of these substrates tested, the uptake of only pantothenate and biotin was significantly increased in the cells transfected with the cDNA in comparison with the vector-transfected cells. The uptake of [³⁵S]pantothenate was typically 4-fold higher in the cDNA-transfected cells compared with the endogenous transport measured in the vector-transfected cells (Fig. 2A) but varied between 3- and 8-fold between
experiments. Replacement of Na\(^+\) with choline in the transport buffer resulted in the complete loss of the uptake activity, demonstrating the Na\(^+\) dependence of the transport process. Significant uptake of Na\(^+\)-dependent pantothenate is also seen in HeLa cells transfected with empty vector, indicating that there is endogenous expression of this transporter in HeLa cells. The substrate specificity of the cloned transporter was analyzed by competition studies. Unlabeled pantothenate, biotin, and lipoate significantly inhibited the uptake of \(^{14}\)C-pantothenate into the cDNA-transfected cells, indicating that all three essential nutrients are substrates for the carrier. Myo-inositol, which is not a substrate of the carrier, failed to inhibit the uptake of the radiolabeled pantothenate.

The substrate specificity of the transporter was also confirmed using \(^{3}\)H-biotin as the radiolabeled substrate. As seen in Fig. 2B, the uptake of \(^{3}\)H-biotin in the presence of Na\(^+\) was stimulated more than 7-fold in the cDNA-transfected cells in comparison with the vector-transfected cells. Replacement of Na\(^+\) with choline in the transport buffer decreased the uptake drastically. The uptake of \(^{3}\)H-biotin was also inhibited by pantothenate and lipoate in addition to unlabeled biotin. Myo-inositol, as in the case of pantothenate uptake, had very little effect on the uptake of \(^{3}\)H-biotin. Thus, the transporter can transport pantothenate, biotin, and lipoate. Based on this substrate specificity, we refer to this new transporter as the sodium-dependent multivitamin transporter (SMVT). The characteristics (Na\(^+\) dependence and substrate specificity) of the cloned transporter are similar to those of the native transporter present in JAR cells and placental brush border membrane vesicles (2–4).

Substrate saturation kinetics of the cloned SMVT were analyzed in cDNA-transfected HeLa cells (Fig. 3). For the kinetic analysis of pantothenate uptake, the concentration of \(^{14}\)C-pantothenate in the uptake buffer was varied from 0.25 to 10 \(\mu\)M. The kinetics of biotin uptake was investigated by measuring the transport over a biotin concentration range of 2.5–100 \(\mu\)M with the \(^{3}\)H-biotin concentration kept constant at 15 nM. Due to the nonavailability of labeled lipoate, the inhibition of \(^{14}\)C-pantothenate and \(^{3}\)H-biotin uptake by lipoate in cDNA-transfected HeLa cells was analyzed. The concentration of lipoate was varied from 0.316 to 31.6 \(\mu\)M. For the uptake of pantothenate and of biotin, the experimental values fit best to a transport model consisting of a single transport system plus a nonsaturable diffusion component. The values for the kinetic constants, \(K_t\) and \(V_{\text{max}}\), for the uptake of pantothenate in cDNA-transfected cells are 4.9 \(\pm\) 1.1 \(\mu\)M and 0.93 \(\pm\) 0.2 nmol/10⁶ cells/30 min, respectively. The corresponding values for the vector-transfected cells are 1.6 \(\pm\) 0.5 \(\mu\)M and 46.1 \(\pm\) 8.6 pmol/10⁶ cells/30 min, respectively.
TABLE I  
Homology of rSMVT to the known members of the sodium-dependent glucose transporter family

<table>
<thead>
<tr>
<th>Protein</th>
<th>Similarity</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli pantothenate permease (panF)</td>
<td>53</td>
<td>23</td>
</tr>
<tr>
<td>H. influenzae pantothenate permease (panF)</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>Rat Na+-dependent glucose transporter 1 (SGLT1)</td>
<td>52</td>
<td>25</td>
</tr>
<tr>
<td>Rat Na+-dependent glucose transporter 2 (SGLT2)</td>
<td>51</td>
<td>22</td>
</tr>
<tr>
<td>Rat Na+-dependent iodide transporter (NIT)</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>Rat Na+-dependent nucleoside transporter (cNT1)</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td>Rat Na+-dependent nucleoside transporter (SPNT)</td>
<td>44</td>
<td>19</td>
</tr>
<tr>
<td>Human myo-inositol transporter</td>
<td>50</td>
<td>23</td>
</tr>
</tbody>
</table>

$10^6$ cells/30 min, respectively. The $K_v$ and $V_{\text{max}}$ values for biotin uptake are $15.1 \pm 1.2 \text{ M}$ and $1.3 \pm 0.1 \text{ mmol/10^6 cells/30 min}$, respectively, in cDNA-transfected cells and $5.8 \pm 1.8 \text{ M}$ and $37.2 \pm 5.1 \text{ pmol/10^6 cells/30 min}$, respectively, in vector-transfected cells. The $IC_{50}$ value (i.e. the concentration necessary to cause 50% inhibition) of lipoate is $7.0 \pm 1.5 \text{ M}$ in the case of pantothenate uptake and $6.4 \pm 1.1 \text{ M}$ in the case of biotin uptake in cDNA-transfected cells. The corresponding values in vector-transfected cells are $4.6 \pm 1.1$ and $2.5 \pm 0.3 \text{ M}$. The inhibition constants ($K_v$) for lipoate, determined using these values according to the method of Cheng and Prusoff (18) are $5.0 \pm 1.1 \text{ M}$ for pantothenate uptake and $6.4 \pm 1.1 \text{ M}$ for biotin uptake in the cDNA transfected cells. Thus the affinity of SMVT for the different substrates is in the following order: pantothenate $>$ lipoate $>$ biotin.

The apparent broad substrate specificity of this Na$^+$-dependent vitamin transporter may have physiological importance. To understand the molecular basis for the ability of the transporter to recognize pantothenate, biotin, and lipoate as substrates, we compared the three-dimensional structure of these three essential nutrients. All three molecules possess a long side chain containing a carboxylate group. Biotin and lipoate possess ring structures. Even though such a ring structure is not apparent in pantothenate, it is possible that hydrogen bonding between the amino group of the $\beta$-alanine moiety and the hydroxyl group in the pantoic acid moiety of the molecule may induce a ring conformation. It is known that both biotin and lipoate bind to avidin (19), and apparently a significant similarity in the molecular structure of these essential metabolites provides a rational basis for these findings. Thus the structural domains of pantothenate, biotin, and lipoate that bear significant similarity are most likely involved in the substrate recognition of the cloned transporter. The ability of this transporter to mediate the cellular uptake of three different essential nutrients may have biologic importance. Pantothenate as a component of coenzyme A, biotin as a coenzyme for carboxylation reactions, and lipoate as a component in the oxidative decarboxylation of pyruvate and $\alpha$-ketoglutarate and also as an antioxidant are crucial for viable metabolic function in all cells. As the transport protein responsible for the cellular uptake of these three essential nutrients, SMVT plays a very important physiological function.

Sequence Homology of SMVT with Other Transporters—Sequence comparison with the sequences in the SwissProt data base shows that SMVT exhibits significant homology with some of the other known sodium-dependent nutrient transporters. Of these, the pantothenate permease from E. coli (20) and Hemophilus influenzae (21) and the mammalian iodide transporter, glucose transporters, and myo-inositol transporter show maximal homology with SMVT. The percentage of similarity and identity values obtained from such a comparison are presented in Table I. Interestingly, the pantothenate permeases from E. coli and H. influenzae are functionally similar to SMVT. The pantothenate permease (panF) gene of E. coli has been functionally expressed following cloning (20). The gene codes for a protein that catalyzes the sodium-dependent uptake of pantothenate into the bacterium. The permease gene of H. influenzae was identified by homology search. Similar to the mammalian SMVT, the bacterial and viral pantothenate permeases are integral membrane proteins with 12 putative transmembrane domains. The bacterial protein is specific to pantothenate with a $K_v$ of 0.4 M (22), which is lower in comparison with the mammalian counterpart, which has a $K_v$ of 2–8 M. However, it is not known whether biotin and lipoate are also transported by the bacterial and viral pantothenate permeases. It is interesting to note that rCGAT, which does not belong to the Na$^+$-dependent glucose transporter family, has 46% similarity and 20% identity with rSMVT at the protein level. This probably explains why the rCGAT cDNA probe hybridized to the rSMVT cDNA during the library screening.

Tissue Distribution of SMVT Transcripts—Poly(A)$^+$ RNA isolated from several tissues of rat was analyzed by Northern blot hybridization for the presence of mRNA transcripts of SMVT (Fig. 4). Transcripts were detected in all of the tissues analyzed. The size of the primary transcript was 3.2 kb. An additional minor hybridizing transcript of 6.5 kb was also seen in all tissues. Quantitatively, the absorptive tissues like the liver, brain, lung, and skeletal muscle have very high amounts of the SMVT-specific mRNA. Significant amounts of SMVT transcripts are seen in other tissues such as the liver, brain, lung, heart, and skeletal muscle. This is not surprising because SMVT transports three different vitamins that are obligatory for the proper metabolic functioning of every cell.

In conclusion, we have isolated a cDNA (rSMVT) from a rat placental cDNA library that when expressed in HeLa cells induces Na$^+$ gradient-dependent uptake of pantothenate, biotin, and lipoate. The functional characteristics of the induced activity are similar to those of the pantothenate/biokinase transport activity described in the placenta and JAR cells. Tissue distribution of the SMVT transcripts suggests that this protein may be involved in the entry of pantothenate, biotin, and lipoate in all cell types. Homology search indicates that SMVT gene belongs to the sodium-dependent glucose transporter family.

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