Human Placental Sodium-Dependent Vitamin C Transporter (SVCT2): Molecular Cloning and Transport Function

D. Prasanna Rajan,* Wei Huang,* Binita Dutta,† Lawrence D. Devoe,† Frederick H. Leibach,* Vadivel Ganapathy,*† and Puttur D. Prasad*†‡
†Department of Obstetrics and Gynecology and *Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30912

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We report here on the cloning and functional characterization of human SVCT2, a sodium-dependent vitamin C (ascorbate) transporter. The hSVCT2 cDNA obtained from a human placental choriocarcinoma cell cDNA library, codes for a protein of 650 amino acids with a predicted molecular mass of 70 kDa. At the level of amino acid sequence, the human SVCT2 exhibits 95% identity to its rat homolog. When functionally expressed in mammalian cells, hSVCT2 induces the transport of ascorbic acid. The transport process induced by hSVCT2 is Na⁺-dependent and is specific for ascorbate. The Michaelis-Menton constant (Kₘ) for the transport of ascorbate in cDNA-transfected cells is 69 ± 5 μM. The relationship between the cDNA-specific uptake rate of ascorbate and Na⁺ concentration is sigmoidal with a Na⁺:ascorbate stoichiometry of 2:1. Northern blot analysis shows that SVCT2-specific transcripts are present in heart, brain, placenta, and liver and is absent in lung and skeletal muscle. The size of the principal transcript is ~7.5 kb.

Ascorbic acid (vitamin C) is a cofactor in several metabolic reactions, but, humans do not have the ability to synthesize this vitamin endogenously. This vitamin exists in two chemically distinct forms under physiological conditions, the reduced, ionic ascorbate and the oxidized, nonionic dehydroascorbic acid. Ascorbate plays an essential role in the synthesis of extracellular matrix proteins (1) and therefore is highly essential for the normal growth and proper bone formation of the fetus. The fetal demand of this vitamin is solely met by the transplacental transfer from the maternal circulation to the fetal circulation (2). The mechanism of transport of vitamin C has been extensively studied. One of the mechanisms suggested to operate in the entry of the vitamin into the placenta is its uptake as dehydroascorbic acid mediated by the sodium-independent glucose transporters, GLUT-1, and GLUT-3 (3, 4). However, since the concentration of dehydroascorbic acid in maternal circulation is very low (~2 μM) (1) and also since the affinity of GLUTs for dehydroascorbic acid is in the millimolar range (5), the importance of this mechanism in placental vitamin C uptake remains questionable. Based on placental perfusion studies (6) and our studies using JAR choriocarcinoma cells in culture (7), a second pathway of vitamin C entry into the placenta is known to operate. The substrate for this transport system is ascorbate rather than dehydroascorbic acid and the transport process is active, energized by an electrochemical Na⁺ gradient.

Recently, Tsukaguchi et al. (8) have cloned two different sodium-dependent vitamin C transporters SVCT1 and SVCT2, from rat kidney and rat brain respectively which mediate electrogenic Na⁺-dependent transport of ascorbate when expressed in Xenopus laevis oocytes. The two proteins exhibit only 65% identity at the amino acid level and also differ in their tissue distribution. While SVCT1 is mainly expressed in epithelial tissues such as intestine, liver and kidney, SVCT2 is expressed in all other tissues that were tested. Placenta was not one of the tissues included in the Northern analysis carried out by Tsukaguchi et al. (8), and hence no information is available on the identity of the ascorbate transporter expressed in the placenta. Here we report on the molecular cloning of a cDNA isolated from a human placental trophoblast cell line, which, when expressed in HRPE cells transports ascorbate in a Na⁺-dependent manner. Comparison of amino acid sequences indicate that the cloned transporter is the human homolog of rat SVCT2. The ascorbate transport process mediated by the human placen-
tial SVCT2 exhibits characteristics similar to the ascorbate transport system known to exist in the human placental choriocarcinoma cell JAR (7).

MATERIALS AND METHODS

Materials. L-[carboxyl-14C]Ascorbic acid (16.7 mCi/mmol), [α-32p]-dCTP (3000 Ci/mmol) and the ready-to-go oligolabeling kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Nitroprure nitrocellulose transfer membranes used in the library screening were purchased from Osmonics (Minnetonka, MN). Lipofectin, used in transfection, and DMEM/F-12 medium and the antibiotics (penicillin and streptomycin) used to culture the human retinal pigment epithelial (HRPE) cells were procured from Life Technologies. The HRPE cell line was originally provided by M. A. Del Monte (W. K. Kellogg Eye Center, Department of Ophthalmology, Ann Arbor, MI) and has been in use in our laboratory for several years. The culture medium for these cells was DMEM/F-12 medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 units/ml streptomycin (9). All other chemicals were of analytical grade.

Screening of the human placental JAR cell cDNA library. A JAR cell cDNA library was screened using human SVCT1 cDNA as the probe. The human SVCT1 cDNA was isolated by screening a Caco2 cell cDNA library using a 1039 bp fragment of rat SVCT1 cDNA obtained by RT-PCR with rat kidney mRNA (manuscript in preparation). This probe corresponded to the nucleotide position 486-1524 in the published sequence of rat SVCT1 cDNA (8). The identity of the amplified DNA was confirmed by sequencing before using it as the probe. The cDNA probe was labeled with [α-32p]dCTP using the ready-to-go oligolabeling kit and used to screen the Caco2 cell cDNA library. A positive clone was selected and the identity was confirmed by partial sequencing. The full length insert was released using restriction enzymes SalI/XbaI and was used to screen the JAR cell cDNA library as described before (10, 11).

DNA sequencing. Both sense and antisense strands of the cDNA were sequenced by primer walking using Taq Dye-Deoxy terminator cycle sequencing in an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the BCM Search Launcher server at http://dot.imgen.bcm.tmc.edu:9331/ (12) and NCBI server at http://www.ncbi.nlm.nih.gov/.

Functional expression of the cDNA in HRPE cells. The vaccinia virus expression system was used to functionally characterize the cloned cDNA as described previously (10, 11). hSVCT2 was cloned into pSPORT plasmid vector such that the sense transcription is under the control of T7 promoter and the transcription of the cDNA insert was carried out by a recombinant vaccinia virus carrying the gene for T7 RNA polymerase. The cDNA (1 μg/well) was transfected into virus-infected HRPE cells grown in 24-well tissue culture plates using lipofectin and the functional expression of the cDNA was determined 12 h later by measuring radiolabeled ascorbate uptake. Cells transfected identically with empty vector were used as control. The transport buffer was composed of either 25 mM Hepes/Tris (pH 7.5) supplemented with 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose. Dithiothreitol (DTT) (1 mM) was also added to the transport buffer to prevent the oxidation of ascorbic acid. At this concentration, DTT had no effect on the transport process (data not shown). When the effect of Na+ on ascorbate transport was assessed, the NaCl in the buffer was replaced with N-methyl-D-glucamine (NMGD) chloride. When the influence of increasing concentrations of Na+ on ascorbate transport was investigated, two different buffers containing either 140 mM NaCl or 140 mM NMGD chloride were mixed to give transport buffers of desired Na+ composition. The incubation time for the transport measurements was 30 min at 37°C, following which the uptake medium containing the radioactive substrate was aspirated off and the cells were washed with 2 × 2 ml of ice-cold transport buffer. The cells were then solubilized in 0.5% SDS in 0.2 N NaOH, transferred to vials and radioactivity associated with the cells was quantitated by liquid scintillation spectrometry. In experiments dealing with saturation kinetics, data were analyzed by nonlinear regression and confirmed by linear regression. The experiments were repeated 2-4 times, each done in duplicate or triplicate. Data are presented as means ± SEM of these replicate measurements.

Northern blot analysis. A commercially available Northern blot (Clontech) containing 2 μg of mRNA isolated from different human tissues was used to determine the expression of hSVCT2 transcripts in various tissues. The filter was sequentially probed, first with the hSVCT2 cDNA probe obtained by digesting hSVCT2 cDNA with SalI and SpeI, followed by the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA-specific probe. Both the probes were labeled with [α-32p]dCTP by random priming using the ready-to-go oligolabeling kit. The hybridization and post-hybridization washings were done under high stringency conditions.

RESULTS AND DISCUSSION

Isolation of hSVCT2 cDNA from a JAR cell cDNA library. Recently, Tsukaguchi et al. (8) reported on the cloning of two cDNAs from rat tissues which, when expressed in X. laevis oocytes, induced Na+-dependent uptake of ascorbate. These cDNAs were referred to as rat SVCT1 and rat SVCT2. Both the proteins are structurally similar but differ in their tissue distribution. We had previously reported the presence of a sodium-dependent ascorbate transporter in the JAR human placental cell line (7). It was therefore of interest to see which of the two ascorbate transporters described by Tsukaguchi et al. (8) is expressed in the human placental cell line. Since the intestine was shown to express SVCT1, we first isolated the human homolog of SVCT1 from a Caco2 cell (a human intestinal cell line) cDNA library using a fragment of the rat SVCT1 as the probe. The cDNA probe was obtained by designing primers using the published sequence of rat SVCT1 cDNA followed by RT-PCR of poly(A)+ RNA isolated from rat kidney. A single positive clone was obtained with a cDNA insert of ~2.2 kb. Sequencing of this clone showed that the cloned transporter was the human homolog of rat SVCT1 (manuscript under preparation). The human SVCT1 cDNA was next used to screen a JAR cell cDNA library. Once again, a single positive clone was identified with a cDNA insert size of ~4.2 kb. Initial sequencing of the 5’-end of this clone indicated that it had the putative translation initiation site and hence was used for further characterization.

Structure of hSVCT2. The cDNA is 4238 bp long (GenBank Accession No. AF164142) with a single open reading frame of 1953 bp, including the termination codon. The open reading frame, which is flanked by a 5’-non-coding region of 393 bp and a 3’-non-coding region of 1892 bp, encodes a 650-amino acid protein with a relative molecular mass of 70,337 Daltons (Fig. 1). Kyte-Doolittle hydropathy analysis (13) of the amino acid sequence predicts a topographical model of
FIG. 1. hSVCT cDNA and the predicted primary amino acid sequence. Putative transmembrane domains are underlined and putative N-linked glycosylation sites are boxed.
hSVCT2 with 12 potential transmembrane domains, with both the N-terminus and the C-terminus towards the intracellular side. Both the N-terminal (102 amino acids) and C-terminal (81 amino acid) tails facing the cytoplasmic side are extremely long and highly hydrophilic. The extracellular loop between the transmembrane domains 3 and 4 contains two potential sites for N-glycosylation (Asn-188 and Asn-196). There are also five potential sites for protein kinase C-dependent phosphorylation (Thr-9, Ser-299, Ser-455, Ser-513 and Thr-629) in putative intracellular domains.

A Blast search (14) of the GenBank sequence data-base using the amino acid sequence of hSVCT2 revealed that two groups of investigators have already independently cloned the protein, however, without knowing the functional identity of the protein. This protein has been referred to as the yolk sac permease-like molecule 2 (15) or the nucleobase transporter-like 1 protein (Hogue, D. L. and Ling, V., direct submission to GenBank). The Blast search also revealed that SVCT2 is highly conserved across the species and exhibits 95-98% identity with the homologous proteins from rat, mouse and rabbit. With the only other known member of the vitamin C transporter family (SVCT1), SVCT2 shares an identity of 65% and a similarity of 79% at the level of amino acid sequence.

**Functional expression of hSVCT2.** To analyze the transport function of hSVCT2, we used the vaccinia virus expression system. We expressed functionally the hSVCT2 cDNA in HRPE cells and measured the uptake of $[^{14}C]$ascorbate at different time intervals (Fig. 2). There was a several-fold stimulation of ascorbate uptake in SVCT2-transfected cells in comparison to empty-vector transfected cells when the uptake measurements were made in the presence of Na$^{+}$. At 30 min, the uptake measured in SVCT2-transfected cells was ~8-fold higher compared to pSPORT-transfected cells. Removal of Na$^{+}$ from the transport buffer abolished the SVCT2-induced transport activity almost completely demonstrating the Na$^{+}$-dependence of the transport process. The uptake was also found to be linear up to 60 min ($r^2 = 0.97$), and hence all subsequent experiments were done with a 30-min incubation.

The ionic dependence of the SVCT2-stimulated ascorbate uptake was investigated by measuring ascorbate transport in empty vector- and SVCT2 cDNA-transfected cells in the presence of various inorganic salts (Table I). Control uptake was measured in the presence of Na$^{+}$. Replacement of Na$^{+}$ with other cat-

**TABLE I**

<table>
<thead>
<tr>
<th>Inorganic salt</th>
<th>pSPORT</th>
<th>pSPORT-cDNA</th>
<th>cDNA-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.03 ± 0.10 (100)</td>
<td>5.93 ± 0.78 (100)</td>
<td>4.9 (100)</td>
</tr>
<tr>
<td>Na gluconate</td>
<td>0.87 ± 0.06 (85)</td>
<td>5.39 ± 0.59 (91)</td>
<td>4.5 (92)</td>
</tr>
<tr>
<td>LiCl</td>
<td>0.03 ± 0.01 (3)</td>
<td>0.09 ± 0.01 (2)</td>
<td>0.06 (1)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.05 ± 0.01 (5)</td>
<td>0.04 ± 0.01 (1)</td>
<td>0.00 (0)</td>
</tr>
</tbody>
</table>

Note HRPE cells transfected with either empty vector or hSVCT2 cDNA were incubated with $60 \text{ nM}[^{14}C] \text{ascorbate}$ for 30 min at room temperature either in the control buffer (25 mM Hepes/Tris, pH 7.5, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, 5 mM glucose, 1 mM DTT and 140 mM NaCl) or in buffers in which NaCl was replaced with 140 mM of various inorganic salts. When the influence of replacement of Cl$^{-}$ with gluconate was studied, KCl and CaCl$_2$ in the buffer were also replaced with potassium gluconate and calcium gluconate, respectively. After incubation for 30 min at 37°C, the cells were washed with the respective buffer (ice-cold) and the radioactivity associated with the cells was quantitated. Uptake measured in control cells transfected with empty vector was subtracted to obtain cDNA-specific uptake. Values in parentheses are percent of corresponding control uptake. Data represent means ± S.E. from three independent determinations.
ions such as K\(^+\) and Li\(^+\) almost completely abolished the ascorbate uptake in both vector- and SVCT2-transfected cells, indicating that Na\(^+\) is obligatory for the transport function. Such a marked inhibition was not seen when Cl\(^-\) in the buffer was replaced with gluconate, suggesting that Cl\(^-\) ions are not obligatory for the transport process.

The substrate specificity of the transporter was evaluated by assessing the ability of various unlabeled vitamins to inhibit the transport of radiolabeled ascorbate in pSPORT-transfected and pSPORT-cDNA-transfected cells (Table II). Only unlabeled ascorbate was able to inhibit the uptake of radiolabeled ascorbate indicating that the transporter is specific for ascorbate. The other vitamins pantothenate, biotin, lipoate, nicotinic acid and thiamine had no or little effect on the uptake of radiolabeled ascorbate. These results are similar to those obtained in ascorbate uptake studies using JAR choriocarcinoma cells (7).

The kinetics of ascorbate uptake mediated by hSVCT2 was analyzed by measuring ascorbate uptake in HRPE cells transfected with hSVCT2 cDNA. Uptake measurements were made at pH 7.5 and in the presence of Na\(^+\). Initial uptake rates were obtained over the concentration range of 2.5–200 \(\mu\)M ascorbate with a 30 min incubation. Cells transfected with vector alone served as the control for endogenous ascorbate uptake activity. The kinetic constants were initially calculated from the data by non-linear regression (Fig. 3) and confirmed by linear regression (Fig. 3, inset). The transport process was saturable in control cells as well as in cells expressing hSVCT2. In both instances, the Michaelis-Menten constant (\(K_v\)) for the transport process was comparable (62 \(\pm\) 5 \(\mu\)M in cells expressing hSVCT2 and 84 \(\pm\) 24 \(\mu\)M in control cells). However, the maximal velocity (\(V_{\text{max}}\)) increased 6.5-fold (18.7 \(\pm\) 0.5 vs. 2.9 \(\pm\) 0.3 nmol/10\(^6\) cells/30 min) in hSVCT2 expressing cells compared to control cells. These data suggest that HRPE cells express an ascorbate transport system that is kinetically similar to the transport process mediated by hSVCT2. The \(K_v\) value for the cDNA-specific transport was 69 \(\pm\) 5 \(\mu\)M. This value is about 3-fold higher than the value obtained for ascorbate transport in JAR cells (7). The reasons for this difference in the \(K_v\) value between the cloned transporter and the native transporter are not known, but post translational modifications such as glycosylation may play a role in these functional differences.

The effect of Na\(^+\) on the kinetics of ascorbate transport was investigated by measuring the uptake of ascorbate in HRPE cells transfected with hSVCT2 cDNA in the presence of varying concentrations of extracellular Na\(^+\). The concentration of NaCl in the ex-

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**TABLE II**

Substrate Specificity of hSVCT2

<table>
<thead>
<tr>
<th>Substrate analog</th>
<th>pSPORT</th>
<th>pSPORT-cDNA</th>
<th>cDNA-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.15 (\pm) 0.05 (100)</td>
<td>5.77 (\pm) 0.33 (100)</td>
<td>4.62 (100)</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.14 (\pm) 0.01 (12)</td>
<td>0.64 (\pm) 0.04 (11)</td>
<td>0.50 (11)</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>1.16 (\pm) 0.03 (101)</td>
<td>5.30 (\pm) 0.12 (92)</td>
<td>4.14 (90)</td>
</tr>
<tr>
<td>Biotin</td>
<td>1.14 (\pm) 0.02 (99)</td>
<td>6.48 (\pm) 0.07 (112)</td>
<td>5.34 (116)</td>
</tr>
<tr>
<td>Lipoate (reduced)</td>
<td>0.86 (\pm) 0.04 (75)</td>
<td>6.45 (\pm) 0.07 (112)</td>
<td>5.59 (121)</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.03 (\pm) 0.02 (90)</td>
<td>4.94 (\pm) 0.03 (86)</td>
<td>3.91 (85)</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1.21 (\pm) 0.07 (105)</td>
<td>5.39 (\pm) 0.15 (93)</td>
<td>4.18 (90)</td>
</tr>
</tbody>
</table>

Note. HRPE cells were transfected with either pSPORT alone or pSPORT-hSVCT2 cDNA. Transport of \([^{14}C]\) ascorbate (60 nM) was measured in these cells with a 30 min incubation at 37°C in the absence or presence of indicated vitamins (1 mM). Values in parentheses are percent of corresponding control uptake. Data represent means \(\pm\) S.E. for three to six determinations.
tracellular medium was varied over a range of 0–117 mM. The uptake was measured in control cells transfected with vector alone as well as in cells expressing hSVCT2. The hSVCT2-specific uptake was determined by subtracting the uptake measured in control cells from the uptake measured in for the hSVCT2-specific uptake. The relationship between the uptake rate and the Na\(^+\) concentration was sigmoidal (Fig. 4), suggesting the involvement of more than one Na\(^+\) per ascorbate molecule transported. The data were fit to the Hill equation and the Hill co-efficient, which is the number of Na\(^+\) ions interacting with the carrier, was calculated. The value was 1.9 for the uptake of ascorbate. This value was confirmed from the slope of the Hill plot (Fig. 4, inset). This indicates that, for every ascorbate molecule transported, 2 Na\(^+\) ions are cotransported. Since ascorbate exists as a monovalent anion at physiological pH, the transport process is electrogenic. Thus, both the Na\(^+\) gradient as well as the difference in the membrane potential across the cell membrane energize the transport process.

Expression of hSVCT2 in various tissues of human origin. This was investigated by Northern blot analysis using a commercially available multiple tissue blot (Fig. 5). The placental mRNA showed two hybridization signals, a more intense signal corresponding to 7.5 kb in size and a less intense signal corresponding to 4.0 kb in size. Heart, brain and liver showed only a single hybridization signal, 7.5 kb in size. The hybridization signal was most intense in the lane containing poly(A)\(^+\) RNA from brain and the hybridization signal in the lane containing poly(A)\(^+\) RNA from heart was weak but clearly visible. No SVCT2-specific transcripts were detected in lung and skeletal muscle.

To summarize, we have reported here on the isolation of the human SVCT2 cDNA from a placental cell line cDNA library, which when expressed in HRPE cells induces Na\(^+\) gradient-dependent uptake of ascorbate. The hSVCT2-mediated transport process is electrogenic with a Na\(^+\):ascorbate stoichiometry of 2:1. The functional characteristics of the induced transport activity are similar to those described for ascorbate transport activity in JAR cells. SVCT2-specific transcripts are detectable in humans in several tissues, with the notable exception of lung and skeletal muscle.

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REFERENCES


