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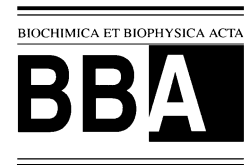

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Rapid report

Human Na^+-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant

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Abstract

We report here on the cloning and functional characterization of human Na^+-dependent vitamin C transporter 1 (SVCT1). The human SVCT1 cDNA, obtained from a Caco2 cell cDNA library, encodes a protein of 598 amino acids with 12 putative transmembrane domains. The SVCT1-specific transcript, 2.4 kb in size, is expressed in kidney, liver, small intestine, thymus and prostate. When expressed heterologously in HRPE cells, SVCT1 mediates the transport of ascorbate, the reduced form of vitamin C, in a Na^+-dependent manner. The transporter is specific for ascorbate with a K_t of ~75 μM. The relationship between the cDNA-specific uptake rate of ascorbate and Na^+ concentration is sigmoidal with a Na^+:ascorbate stoichiometry of 2:1, indicating that the transport process is electrogenic. In Caco2 cells and in normal human intestine, SVCT1 also exists as a non-functional splice variant with a four amino acid sequence inserted between E-155 and V-156. The splice variant results from the use of a donor site 12 bp downstream of the normal donor site. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ascorbate transport; Na^+-dependent vitamin C transporter 1; Primary structure; Na^+-dependent vitamin C transporter 1 splice variant; Intestine; Human

Ascorbate (vitamin C), an essential cellular nutrient, is involved in a variety of metabolic reactions and is an important reducing agent in biological systems [1–3]. While certain animals like the rat and rabbit are capable of endogenous synthesis of ascorbate from glucose, humans depend on the dietary supply to meet their requirements for this anti-oxidant. Two types of membrane transport activity have been described for entry of vitamin C into mammalian cells: transport by a high-affinity/low-capacity active carrier, as well as a low-affinity/high-capacity facilitative carrier. The high-affinity carrier is a Na^+-dependent transport system that accepts only the reduced ascorbate as the substrate [4–8]. The low-affinity carrier is a Na^+-independent system mediated by the facilitative glucose transporters (GLUTs) [9,10]. The substrate for this system is the oxidized, non-ionic form (dehydroascorbic acid, DHAA) of vitamin C. Once inside the cell, the DHAA is rapidly

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reduced to ascorbic acid, providing a mechanism for its concentrative accumulation inside the cell. However, since the vitamin is predominantly present in its reduced form under physiological conditions [11] and also since the GLUTs have a higher affinity for glucose than for DHAA [12], it is highly unlikely that GLUT-mediated vitamin C transport will suffice the cellular needs for the vitamin.

Recently, Tsukaguchi et al. [13] have isolated two ascorbate transporters, Na^+-dependent vitamin C transporter (SVCT) 1 and SVCT2, from rat kidney and rat brain, respectively. Both the transporters mediate, when expressed in *Xenopus* oocytes, Na^+-dependent electrogenic transport of ascorbate. The two proteins share an amino acid sequence identity of 65% and show differential tissue distribution. While SVCT1 is restricted to absorptive tissues (intestine and kidney) and the liver, SVCT2 is more widely distributed.

In this paper, we report on the cloning of the human SVCT1 (hSVCT1) cDNA from a Caco2 cell (a human intestinal cell line) cDNA library. We have expressed this cDNA in HRPE cells by the vaccinia virus expression technique and characterized the transport function mediated by the cDNA. Similar to that seen in the rat, the hSVCT1 is expressed in kidney, intestine and liver as demonstrated by Northern analysis and dot-blot analysis of mRNA isolated from different tissues of human origin. We also provide evidence for the presence of a non-functional splice variant of SVCT1 in Caco2 cells and furnish the molecular basis for the generation of the splice variant.

The SuperScript Plasmid system (Gibco BRL) was used to establish a unidirectional Caco2 cell cDNA library. The rat SVCT1-specific probe used in the screening of the cDNA library was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using poly(A)$^+$ RNA isolated from rat kidney. The primers used were 5′-CGGGAGGTCCAGGGTGCAATCATGGT-3′ and 5′-AGACAGCCACCAACAAACATCTC-3′, designed based on the published sequence of rat SVCT1 [13]. A single product was obtained with an estimated size of 1061 bp (from nucleotide 486 to 1546 of rat SVCT1) as predicted by the primers. The PCR product was cloned into pGEM-T easy vector (Promega) and sequenced to confirm the identity of the PCR product before using it as the probe. The probe was labelled with [α-^{32}P]dCTP by random priming using the ready-to-go oligolabelling kit (Amersham Pharmacia Biotech) and used to screen the Caco2 cell cDNA library as described before [14,15]. A single positive clone was identified which was purified by secondary screening.

The expression of SVCT1 in different tissues was determined by Northern analysis. A commercially available blot with poly(A)$^+$ RNA isolated from eight different human tissues (Clontech, cat# 7760-1) was probed with hSVCT1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes under high stringency conditions. A second blot with RNA from 43 different adult human tissues and seven different fetal tissues spotted on a nylon filter (Clontech, cat# 7770-1) was also probed with hSVCT1-specific probe under high stringency conditions. The probes were labelled with [α-^{32}P]dCTP by random priming using the ready-to-go oligolabelling kit.

Functional expression of the hSVCT1 cDNA was done using the vaccinia virus expression system in HRPE cells as described before [15,16]. Subconfluent HRPE cells grown on 24 well plates were first infected with a recombinant (VTF_{7-3}) vaccinia virus encoding T7 RNA polymerase and then transfected with the plasmid carrying the full-length cDNA. Twelve hours post-transfection, uptake measurements were made at 37°C with [^{14}C]ascorbate. The transport buffer was 25 mM HEPES/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. In addition, the transport buffer was supplemented with 1 mM dithiothreitol (DTT) to maintain the ascorbate under reduced state. The uptake was carried out by incubating the cells with the substrate for 5 min. This time was chosen because initial time course experiments showed that the uptake was linear up to

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Fig. 1. hSVCT1 cDNA and the predicted primary amino acid sequence. Putative transmembrane domains are underlined, putative *N*-linked glycosylation sites are boxed and putative phosphorylation sites are circled. The point of 12 bp insertion is marked with an arrow.

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   1 CTCAGGAACTGCTCAAACCTGTGCCCCAAAGATGAGGGCCCAAGAGGACCTCGAGGGCCG
                                      M  R  A  Q  E  D  L  E  G  R    10
  61 GGCACAGCATGAAACCACCAGGGACCCCTCGACCCCGCTACCCACAGAGCCTAAGTTTGA
      A  Q  H  E  T  T  R  D  P  S  T  P  L  P  T  E  P  K  F  D    30
 121 CATGTTGTACAAGATCGAGGACGTGCCACCTTGGTACCTGTGCATCCTGCTGGGCTTCCA
      M  L  Y  K  I  E  D  V  P  P  W  Y  L  C  I  L  L  G  F  Q    50
 181 GCACTACCTGACATGCTTCAGTGGTACCATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCT
      H  Y  L  T  C  F  S  G  T  I  A  V  P  F  L  L  A  E  A  L    70
 241 GTGTGTGGGCCACGACCAGCACATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGT
      C  V  G  H  D  Q  H  M  V  S  Q  L  I  G  T  I  F  T  C  V    90
 301 GGGCATCACCACTCTCATCCAGACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAG
      G  I  T  T  L  I  Q  T  T  V  G  I  R  L  P  L  F  Q  A  S   110
 361 TGCCTTTGCATTTCTGGTTCCAGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCC
      A  F  A  F  L  V  P  A  K  A  I  L  A  L  E  R  W  K  C  P   130
 421 CCCGGAAGAGGAGATCTACGGTAACTGGAGTCTGCCCCTGAACACCTCTCATATTTGGCA
      P  E  E  E  I  Y  G  N  W  S  L  P  L  N  T  S  H  I  W  H   150
 481 CCCACGGATACGGGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGTGAT
      P  R  I  R  E  V  Q  G  A  I  M  V  S  S  V  V  E  V  V  I   170
 541 TGGCCTGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCACAGTCACCCC
      G  L  L  G  L  P  G  A  L  L  N  Y  I  G  P  L  T  V  T  P   190
 601 CACTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGCTCCCA
      T  V  S  L  I  G  L  S  V  F  Q  A  A  G  D  R  A  G  S  H   210
 661 CTGGGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACCTGCGCAA
      W  G  I  S  A  C  S  I  L  L  I  I  L  F  S  Q  Y  L  R  N   230
 721 CCTCACCTTCCTGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCCTCCGCATCCA
      L  T  F  L  L  P  V  Y  R  W  G  K  G  L  T  L  L  R  I  Q   250
 781 GATCTTCAAAATGTTTCCTATCATGCTGGCCATCATGACCGTGTGGCTGCTCTGCTATGT
      I  F  K  M  F  P  I  M  L  A  I  M  T  V  W  L  L  C  Y  V   270
 841 CCTGACCTTGACAGACGTGCTGCCCACAGACCCAAAAGCCTATGGCTTCCAGGCACGAAC
      L  T  L  T  D  V  L  P  T  D  P  K  A  Y  G  F  Q  A  R  T   290
 901 CGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCCGCATCCCCTACCCCTGTCA
      D  A  R  G  D  I  M  A  I  A  P  W  I  R  I  P  Y  P  C  Q   310
 961 GTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGGAATGTTCAGCGCCACTCTGGC
      W  G  L  P  T  V  T  A  A  A  V  L  G  M  F  S  A  T  L  A   330
1021 AGGCATCATTGAGTCCATCGGAGATTACTACGCCTGTGCCCGCCTGGCTGGTGCACCACC
      G  I  I  E  S  I  G  D  Y  Y  A  C  A  R  L  A  G  A  P  P   350
1081 CCCTCCAGTACATGCTATCAACAGGGGCATCTTCACCGAAGGCATTTGCTGCATCATCGC
      P  P  V  H  A  I  N  R  G  I  F  T  E  G  I  C  C  I  I  A   370
1141 GGGGCTATTGGGCACGGGCAACGGGTCCACCTCGTCCAGTCCCAACATTGGCGTCCTGGG
      G  L  L  G  T  G  N  G  S  T  S  S  S  P  N  I  G  V  L  G   390
1201 AATTACCAAGGTGGGCAGCCGGCGCGTGGTGCAGTATGGTGCGGCTATCATGCTGGTCCT
      I  T  K  V  G  S  R  R  V  V  Q  Y  G  A  A  I  M  L  V  L   410
1261 GGGCACCATCGGCAAGTTCACGGCCCTCTTCGCCTCGCTCCCTGACCCCATCCTGGGGGG
      G  T  I  G  K  F  T  A  L  F  A  S  L  P  D  P  I  L  G  G   430
1321 CATGTTCTGCACTCTCTTTGGCATGATTACAGCTGTGGGGCTGTCCAACCTGCAATTTGT
      M  F  C  T  L  F  G  M  I  T  A  V  G  L  S  N  L  Q  F  V   450
1381 GGACATGAACTCCTCTCGCAACCTCTTCGTGCTGGGATTTTCCATGTTCTTCGGGCTCAC
      D  M  N  S  S  R  N  L  F  V  L  G  F  S  M  F  F  G  L  T   470
1441 GCTGCCCAATTACCTGGAGTCCAACCCTGGCGCCATCAATACAGGCATTCTTGAAGTGGA
      L  P  N  Y  L  E  S  N  P  G  A  I  N  T  G  I  L  E  V  D   490
1501 TCAGATTCTGATTGTGCTGCTGACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCAT
      Q  I  L  I  V  L  L  T  T  E  M  F  V  G  G  C  L  A  F  I   510
1561 ACTTGACAACACAGTGCCAGGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGG
      L  D  N  T  V  P  G  S  P  E  E  R  G  L  I  Q  W  K  A  G   530
1621 GGCTCATGCCAACAGTGACATGTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGAT
      A  H  A  N  S  D  M  S  S  S  L  K  S  Y  D  F  P  I  G  M   550
1681 GGGCATAGTAAAAAGAATTACCTTTCTGAAATACATTCCTATCTGCCCAGTCTTCAAAGG
      G  I  V  K  R  I  T  F  L  K  Y  I  P  I  C  P  V  F  K  G   570
1741 ATTTTCTTCAAGTTCAAAAGATCAGATTGCAATTCCAGAAGACACTCCAGAAAATACAGA
      F  S  S  S  S  K  D  Q  I  A  I  P  E  D  T  P  E  N  T  E   590
1801 AACTGCATCTGTGTGCACCAAGGTCTGAAAAATGACTTCCAGGAAAGGAAGCATGGTATA
      T  A  S  V  C  T  K  V  *                                      598
1861 TAACAGGAAAAGAAAACTACATGGGGAACCAGAAGACCTAAGCCTGAAATCCCAGCCCTG
     CCCCTAACTAACTTCTGTGTAAACTCAGATAAGTCACCTTTCTCTGGGATTCAAATTTTT
1981 GCATCAGTTAAAAAAAAAGGGGTGGGGGGGAATGGGCCAAAGTCTGAGTCTTAGAGACTT
     GTACCAATGTTATGCTATGTCTCTAAATCTTTACTCTCCTAAGTAGACTTGTCAGCATCT
2101 AGGAAGAACAGCTAGAAATTTTCCTCTGTGATATTTTAGACTGCAAGTTGAAAAAAAAAA
     AAAAAAAAAA
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15 min. Transport was terminated by aspiration of the medium containing the radiolabelled substrate, followed by washing two times with 1 ml of ice-cold transport medium. The cells were then solubilized in 0.5 ml of 1% SDS in 0.1 N NaOH and the radioactivity associated with the cells was determined by liquid scintillation spectrometry. Na^+-dependence of the transport process was investigated with the use of a transport medium in which NaCl was isoosmotically substituted with *N*-methyl-D-glucamine (NMDG) chloride. Endogenous transport was determined in parallel using cells transfected with empty vector. The transport values measured in cells transfected with empty vector were subtracted from the corresponding transport values measured in cells transfected with vector cDNA to obtain the cDNA-specific uptake.

A single positive clone was isolated by screening $\sim 1 \times 10^6$ colonies of the Caco2 cell cDNA library with a cDNA insert size of $\sim$2.4 kb in size. Sequence analysis of the clone indicated that it was a full-length clone with a putative translation start site and an open reading frame. Sequence comparison of the deduced amino acid sequence of the cDNA isolated from the Caco2 cell cDNA library with the amino acid sequence of rat SVCT1 indicated that the cDNA isolated from the Caco2 cell cDNA library is the hSVCT1. The two proteins were highly homologous and shared an identity of 89% and similarity of 92%. However, the hSVCT1 isolated from the Caco2 cell cDNA library had an additional four amino acids (VGLH) located, in reference to rat SVCT1, between amino acids Glu^{155} and Val^{156}. Initial attempts to express this cDNA in HRPE cells by a vaccinia virus-dependent expression system indicated that the hSVCT1 clone with the additional four amino acid insert was non-functional. We therefore deleted the 12 bp sequence encoding the four amino acids using the Quick-change site-directed mutagenesis kit (Stratagene) and a mutant oligonucleotide primer pair. The mutant oligonucleotide primers were 5′-CATATTTGGCACCCAAGGATACGGG-AGGTCCAGGGTGCAATCATG-3′ and its reverse complement, with the desired deletion in the middle. The PCR was performed using *Pfu* DNA polymerase and the hSVCT1 cDNA as the template. The deletion in the resultant product was confirmed by sequencing and by its ability to mediate Na^+-dependent transport of ascorbate when expressed in HRPE cells.

The nucleotide sequence (GenBank accession # AF170911) and the deduced amino acid sequence of the functional hSVCT1 are given in Fig. 1. The cDNA is 2170 bp long with a 1797 bp long open reading frame (including the termination codon). The cDNA encodes a protein of 598 amino acids long with a predicted molecular mass of 65.2 kDa. Hydropathy analysis using the Kyte-Doolittle algorithm indicates that the protein is highly hydrophobic with 12 transmembrane domains. When the transmembrane topology of hSVCT1 is modeled similar to other solute transporters with both the N-terminus and C-terminus directed towards the cytoplasmic side, three putative *N*-glycosylation sites (N-138, N-144 and N-230) can be identified in predicted extracellular domains. Putative recognition sites for cAMP-dependent phosphorylation (T-557) and protein kinase C-dependent phosphorylation (T-15, S-396, S-454, S-540 and S-574) can also be recognized in predicted intracellular domains.

Northern analysis using a commercially available multiple tissue RNA blot indicated that the SVCT1-specific transcript ($\sim$2.4 kb in size) is present in liver and kidney (Fig. 2). Heart, brain, placenta, lung, skeletal muscle and pancreas were devoid of any detectable SVCT1-specific transcripts. To assess the general tissue distribution of hSVCT1 transcript in humans, we probed a commercially available multiple tissue RNA master blot. The master blot con-

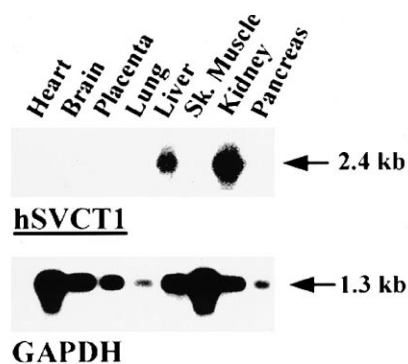

Fig. 2. Northern blot analysis of the hSVCT1-specific transcript in human tissues. The upper panel is the hybridization signal obtained with the hSVCT1 cDNA probe. The lower panel is the hybridization signal obtained with the GAPDH cDNA probe.

tained poly(A)$^+$ RNA from 43 different adult tissues and seven different fetal tissues spotted on a nylon filter. The results obtained indicate that, in addition to kidney and liver, SVCT1 is also expressed abundantly in small intestine, thymus and prostate in adults (data not shown). Among the fetal tissues tested, kidney, liver and thymus are positive for SVCT1-specific transcript.

The functional characterization of the cloned hSVCT1 was done in HRPE cells using the vaccinia virus expression system. The time course of ascorbate transport was studied in cells transfected either with the original cDNA clone containing the 12 bp insertion or with the cDNA clone in which the 12 bp sequence was deleted. The cells transfected with empty vector served as the control. As seen in Fig. 3, cells transfected with the cDNA in which the 12 bp was deleted showed significant stimulation of ascorbate uptake in comparison to the vector-transfected cells. At 5 min incubation, the ascorbate uptake measured in cells transfected with this cDNA was ~7-fold higher than the ascorbate uptake measured in vector-transfected cells. This stimulation was not seen in cells transfected with the cDNA containing the 12 bp insertion. The transport values measured in these cells were comparable to that measured in vector-transfected cells, thereby indicating that the 12 bp insertion, which causes an addition of only four amino acids without altering the sequence of the rest of the protein, results in a non-functional transporter. The hSVCT1-mediated ascorbate uptake was linear up to 15 min and therefore, we have used a 5 min incubation for subsequent initial transport rate measurements.

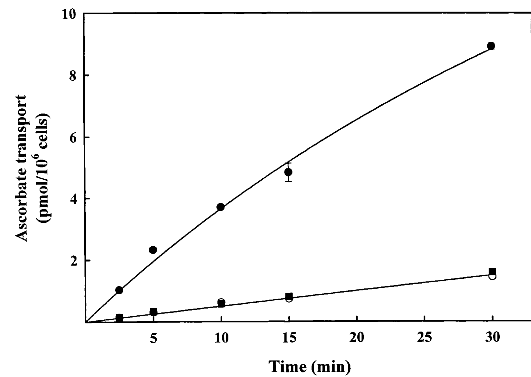

Fig. 3. Time course of ascorbate uptake in control cells transfected with pSPORT (○), in cells transfected with the cDNA isolated from the Caco2 cell cDNA library containing the 12 bp insertion (■) and in cells transfected with the hSVCT1 cDNA from which the 12 bp insertion has been deleted (●). Transport was measured at 37°C in the presence of Na^+. The pH of transport buffer was 7.5 and the concentration of [^{14}C]ascorbate was 60 nM.

The ionic requirements of the cDNA-induced transport activity were investigated by substituting NaCl in the transport buffer with different salts and assessing the resultant effect on the transport of [^{14}C]ascorbate in cDNA-transfected cells (Table 1). Substitution of Na^+ in the transport buffer with Li^+ or K^+ ions abolished the transport completely,

Table 1
Ionic dependence of ascorbate uptake in HRPE cells transiently expressing hSVCT1

Inorganic salt	Ascorbate uptake (pmol/10^6 cells/5 min)		
	pSPORT	pSPORT-cDNA	cDNA-specific
NaCl	0.19 ± 0.02 (100)	1.01 ± 0.18 (100)	0.82 (100)
Na-gluconate	0.20 ± 0.04 (105)	0.89 ± 0.10 (88)	0.69 (84)
LiCl	0.03 ± 0.01 (16)	0.03 ± 0.01 (3)	0 (0)
KCl	0.03 ± 0.01 (16)	0.03 ± 0.01 (3)	0 (0)

HRPE cells were transfected with either empty vector or hSVCT1 cDNA. Twelve hours post-transfection, the cells were incubated with 60 nM [^{14}C]ascorbate for 5 min at 37°C 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 and 140 mM NaCl) or in buffers in which NaCl was replaced with 140 mM of various inorganic salts. The buffer was supplemented with 1 mM DTT to maintain ascorbate under reduced conditions. 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 5 min at 37°C, the cells were washed with the respective buffer (ice-cold) and the radioactivity associated with the cells quantitated. Uptake measured in control cells transfected with empty vector was subtracted to obtain cDNA-specific uptake. Values in parentheses are percentages of corresponding control uptake. Data represent means ± S.E.M. from three replicate measurements.

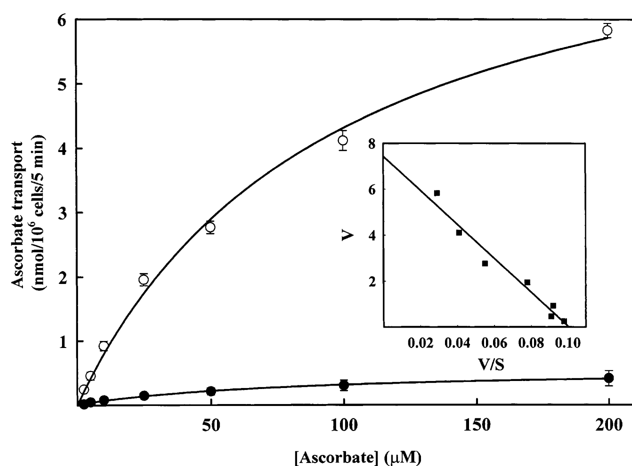

Fig. 4. Saturation kinetics of hSVCT1-mediated ascorbate transport. HRPE cells were transfected with either vector alone (●) or with hSVCT1 cDNA (○). Transport of ascorbate was measured over an ascorbate concentration range of 2.5–200 μM. The concentration of labelled ascorbate was kept constant at 60 nM. Transport was measured for 5 min at 37°C in NaCl-containing buffer, pH 7.5. Inset, Eadie-Hofstee plot of cDNA-specific transport. The transport values measured in cells transfected with empty plasmid were subtracted from values measured in cells transfected with the plasmid-cDNA construct to calculate the cDNA-specific transport. V, ascorbate transport in nmol/10^6 cells/5 min; S, ascorbate concentration in μM.

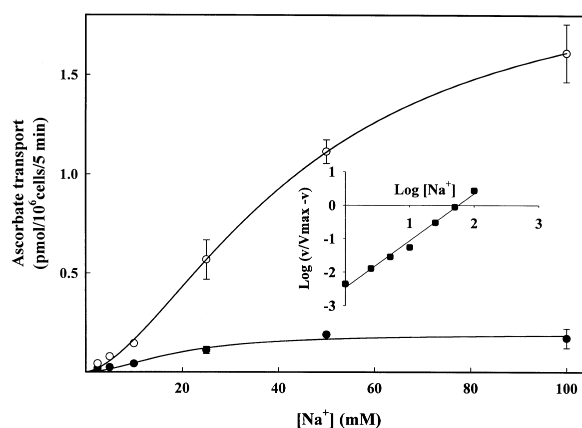

Fig. 5. Na^+ activation kinetics of ascorbate transport in HRPE cells expressing the cloned hSVCT1 cDNA. Transport of ascorbate (60 nM) was studied in HRPE cells transfected with either pSPORT vector (●) or hSVCT1 cDNA (○) in the presence of increasing concentrations of Na^+ (0–110 mM). The concentration of Cl^- in the transport medium was maintained at 140 mM. The osmolality of the medium was kept constant by replacing Na^+ with appropriate concentrations of NMDG. Inset, Hill plot of the cDNA-specific transport. The uptake values measured in cells transfected with empty plasmid were subtracted from values measured in cells transfected with the plasmid-cDNA construct to calculate the cDNA-specific transport. V, uptake rate in pmol/10^6 cells/5 min; V_m, the maximal uptake rate calculated from the experimental data using the Hill equation.

confirming that the transport process is obligatorily dependent on the presence of Na^+. When the Cl^- in the transport buffer was replaced with gluconate, the cDNA-induced transport was only minimally affected, suggesting that Cl^- is not obligatory for the transport process.

The substrate specificity of the cDNA-induced transport activity was investigated by assessing the ability of various vitamins to compete with [^{14}C]ascorbate for the transport process (Table 2). The transport of [^{14}C]ascorbate (35 nM) was inhibited by ~90% by unlabelled ascorbate (1 mM). Oth-

Table 2
Substrate specificity of hSVCT1

Substrate	[^{14}C]Ascorbate uptake (pmol/10^6 cells/5 min)		
	pSPORT	pSPORT-cDNA	cDNA-specific
Control	0.19 ± 0.01 (100)	1.47 ± 0.02 (100)	1.28 (100)
Ascorbate	0.01 ± 0.01 (12)	0.17 ± 0.02 (12)	0.16 (12)
Pantothenate	0.19 ± 0.01 (101)	1.46 ± 0.01 (99)	1.27 (99)
Biotin	0.19 ± 0.01 (99)	1.41 ± 0.02 (96)	1.22 (96)
Lipoate (reduced)	0.14 ± 0.01 (75)	1.46 ± 0.04 (99)	1.32 (103)
Nicotinic acid	0.17 ± 0.01 (90)	1.45 ± 0.02 (99)	1.28 (100)
Thiamine	0.20 ± 0.01 (105)	1.40 ± 0.04 (96)	1.20 (94)

HRPE cells were transfected with either pSPORT alone or pSPORT-hSVCT1 cDNA. Twelve hours post-transfection, transport of [^{14}C]ascorbate (60 nM) was measured in these cells with a 5 min incubation at 37°C in the absence or presence of indicated vitamins (1 mM). Values in parentheses are percentages of corresponding control uptake. Data represent means ± S.E.M. for 3–6 determinations.

A

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  1 ATACGGGAGgtgggtttgcatgtaagcatagaagaggccgggtcctgagg
    aggggctatatgggagtgagggtggccgctgagtttggggatttgaggag
100 gtggtccaggagctttgttcctgctagcagcagccaggtcatgaccttta
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200 TGGTGTCCAGCGTGGTGGAGGTGGTGATTGGCCTGCTGGGGCTGCCTGGG
    GCCCTGCTCAACTACATTGGGCCTCTCACAGTCACCCCCACTGTCTCCCT
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B

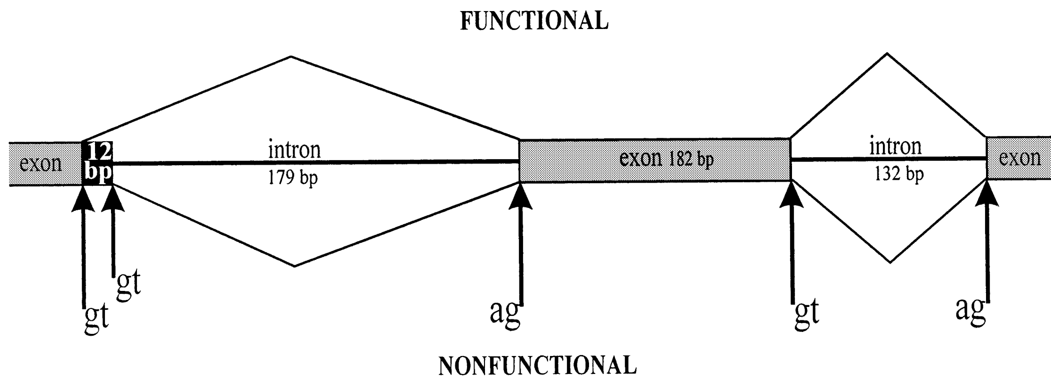

Fig. 6. (A) Nucleotide sequence of the PCR product amplified from the genomic DNA. The letters in uppercase denote exonic sequences and the letters in lowercase denote intronic sequences. The splice donor sites and the acceptor sites are marked in bold. The sequence of the 12 nucleotides present in the splice variant is underlined. (B) Diagram indicating the structure and alternative splicing pattern of hSVCT1 gene.

er vitamins tested (pantothenate, biotin, lipoic acid, nicotinic acid and thiamine) had no effect on [^{14}C]ascorbate transport, showing that hSVCT1 encodes a transporter which is highly specific for ascorbate.

Fig. 4 describes the saturation kinetics of ascorbate transport in HRPE cells mediated by hSVCT1. Transport was measured in vector-transfected cells and in cDNA-transfected cells with varying concentrations of ascorbate (2.5–200 μM). The transport in control cells was subtracted from the transport in cDNA-transfected cells to determine the cDNA-specific transport. The hSVCT1-specific ascorbate uptake was saturable, with a Michaelis-Menten constant of 74 ± 6 μM.

We next studied the kinetics of Na^+ activation of the transport process. Ascorbate transport was measured in HRPE cells expressing hSVCT1 in the presence of varying concentrations of Na^+ (0–100 mM). The cDNA-specific uptake was determined by subtracting the uptake measured simultaneously in cells transfected with pSPORT vector. The relationship between the uptake rate and the Na^+ concentration was sigmoidal for the cDNA-specific transport process (Fig. 5), suggesting the involvement of more than one Na^+ per ascorbate molecule transported. The data were fit to the Hill equation and the Hill coefficient, which is the number of Na^+ ions interacting with the carrier, was calculated. The value for the Hill coefficient was 1.6 ± 0.1, which was confirmed

from the slope of the Hill plot (Fig. 5, inset). This indicates that, for every ascorbate molecule transported, two Na^+ ions are co-transported. Since ascorbate exists as a monovalent anion at physiological pH, the Na^+:ascorbate stoichiometry of 2:1 renders the transport process electrogenic.

The original clone that we isolated from the Caco2 cell cDNA library is a splice variant with a 12 bp insertion. To confirm that the 12 bp insertion was not a cloning artifact and also to understand the molecular basis of the generation of the splice variant, we amplified this region of the human genome by PCR using human genomic DNA isolated from human leukocytes and the PCR primers 5′-ATACGGGAGGTGGGTTTG-3′ (sense) and 5′-GCCCCAGCGGTTAGACAG-3′ (antisense) (Fig. 6A). The underlined sequence in the sense primer represents the first nine nucleotides of the 12 base insert in the clone isolated from the Caco2 cell cDNA library. The antisense primer was 245 bases downstream of the site of the 12 bp insertion. A 578 bp PCR product was obtained which was subcloned and sequenced. Comparison of the nucleotide sequence of the PCR product with the nucleotide sequence of the functional hSVCT1 indicated that the 578 bp PCR product contained two introns (Fig. 6A). Following the 12 nucleotides in the beginning of the first intron are the nucleotides GTAAGCA. This GT can form a potential donor site resulting in the generation of the splice variant with a 12 bp insertion (Fig. 6B). The resulting splice variant is inactive with respect to transport function. RT-PCR using poly(A)$^+$ RNA isolated from normal human intestine also showed the presence of this splice variant in normal enterocytes (data not shown). While it may be too premature to attach any special significance to the occurrence of the splice variant of hSVCT1, recent studies have suggested that alternative splicing may be a mechanism of gene regulation hitherto unknown [17]. The putative protein coded by the splice variant contains a four amino acid sequence (VGLH) inserted between E-155 and V-156 of the functionally active SVCT1. This region lies in the second extracellular loop between the putative transmembrane domains 3 and 4. The molecular mechanism by which this structural alteration results in the loss of transport activity remains to be determined.

In conclusion, we have isolated a cDNA (hSVCT1) from a Caco2 cell cDNA library that, when expressed in HRPE cells, induces Na^+ gradient-dependent uptake of ascorbate. The functional characteristics of the induced activity are similar to those of the ascorbate transport activity described in the intestine. The SVCT1-specific transcript is expressed in the intestine, liver, kidney, thymus and prostate. We have also demonstrated the existence of a non-functional splice variant of SVCT1 in Caco2 cells and in normal human intestine. The splice variant results from the use of the donor site 12 bp downstream of the normal donor site, causing an addition of 12 nucleotides in the resulting mRNA variant.

Acknowledgements

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