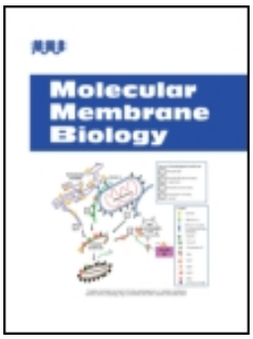

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Vitamin C transport systems of mammalian cells

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Summary

Vitamin C is essential for many enzymatic reactions and also acts as a free radical scavenger. Specific non-overlapping transport proteins mediate the transport of the oxidized form of vitamin C, dehydroascorbic acid, and the reduced form, L-ascorbic acid, across biological membranes. Dehydroascorbic acid uptake is via the facilitated-diffusion glucose transporters, GLUT 1, 3 and 4, but under physiological conditions these transporters are unlikely to play a major role in the uptake of vitamin C due to the high concentrations of glucose that will effectively block influx. L-ascorbic acid enters cells via Na^+-dependent systems, and two isoforms of these transporters (SVCT1 and SVCT2) have recently been cloned from humans and rats. Transport by both isoforms is stereospecific, with a pH optimum of ~7.5 and a Na^+ : ascorbic acid stoichiometry of 2 : 1. SVCT2 may exhibit a higher affinity for ascorbic acid than SVCT1 but with a lower maximum velocity. SVCT1 and SVCT2 are predicted to have 12 transmembrane domains, but they share no structural homology with other Na^+ co-transporters. Potential sites for phosphorylation by protein kinase C exist on the cytoplasmic surface of both proteins, with an additional protein kinase A site in SVCT1. The two isoforms also differ in their tissue distribution: SVCT1 is present in epithelial tissues, whereas SVCT2 is present in most tissues with the exception of lung and skeletal muscle.

Keywords: Ascorbic acid, SVCT1, SVCT2, Na^+-dependent vitamin C transport, dehydroascorbic acid.

Abbreviations: DHA, dehydro-L-ascorbic acid; HRPE, human retinal pigment epithelial cells; PMA, phorbol 12-myristate 13-acetate; SVCTs, Na^+-dependent vitamin C transporters; TM, transmembrane.

Introduction

Vitamin C is an essential nutrient required for the normal metabolic function of the body and exists in two physiological forms as L-ascorbic acid, the reversibly reduced form, and dehydro-L-ascorbic acid (DHA), the reversibly oxidized form (Carr and Frei 1999). The reduced form of vitamin C is a cofactor for several important enzymes acting to maintain transition metal ions in their reduced form and is involved in a variety of metabolic roles including the biosynthesis of extracellular matrix proteins and neurotransmitters, and the regulation of iron uptake (Toth and Bridges 1995, Packer and Fuchs 1997). Ascorbic acid is also a good reducing agent and it is the most important water-soluble antioxidant in human plasma (Frei *et al.* 1989). It effectively scavenges superoxide and other reactive oxygen species and protects lipids against oxidation (Retsky *et al.* 1993, Packer and Fuchs 1997). As such, there has been considerable interest in using vitamin C to affect chronic disease incidence, and much evidence exists to indicate that vitamin C plays a protective role in clinical conditions such as cardiovascular disease, cancer, and cataract formation (Carr and Frei 1999). Deficiency of vitamin C in humans leads to a wide variety of clinical abnormalities, for example, scurvy, poor wound healing, bone and connective tissue disorders and vasomotor instability (Packer and Fuchs 1997).

Humans, primates and guinea pigs have lost the ability to synthesise vitamin C due to the deficiency of L-gulono-γ-lactone oxidase, and, thus, depend on a dietary source in order to survive (Packer and Fuchs 1997). Vitamin C must be absorbed from the small intestine, exit the epithelial cells and be transported to key sites, for example, brain and eye. In addition, the kidney plays an essential role in reabsorbing ascorbic acid from the renal filtrate. At physiological pH, ascorbate is negatively charged, and, although DHA is a non-charged molecule, it is hydrophilic, and specialized transport systems are required for the movement of both compounds across cell membranes. The mechanisms by which vitamin C is transported across plasma membranes are essential for understanding its metabolic role and have been studied in several cell types. This review will focus on the molecular identity of the transporters for DHA and ascorbic acid. In particular, it highlights the functional properties of recently cloned Na^+-dependent ascorbic acid transporters (SVCTs), and the structural information contained in the deduced amino acid sequences of some of the SVCTs.

Uptake of dehydroascorbic acid

Vitamin C exists in two forms, the reduced form and the oxidized form (DHA), and studies on erythrocytes, fibroblasts, HL60 cells and neutrophils have shown that DHA entry involves uptake via a facilitated-diffusion mechanism and its rapid reduction to ascorbic acid intracellularly (Vera *et al.* 1993, 1994, Goldenberg and Schweinzer 1994). DHA is structurally similar to glucose, and its entry into cells can be competitively inhibited by sugars (Vera *et al.* 1995). These findings suggest that entry of DHA is mediated by glucose transporters. To investigate which of the different glucose transporters mediated DHA transport, a *Xenopus laevis* oocyte expression system was used to express the facilitated-diffusion glucose transporter isoforms, GLUT1-5, and the Na^+-dependent glucose transporter, SGLT1. GLUT1 and GLUT3 mediated DHA transport (apparent K_m 1–2 mM) with a similar efficiency to that of glucose (Rumsey *et al.* 1997). GLUT4 also transported DHA with a higher affinity than 2-deoxyglucose (K_m of 0.98 versus 5.2 mM), but with a 13-fold lower V_{max} (Rumsey *et al.* 2000). GLUT2, GLUT5 and SGLT1 did not transport DHA, and none of the glucose transporter isoforms (GLUT1–5 and SGLT1) transported ascorbic acid (Rumsey *et al.* 1997).

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Under physiological conditions, the reduced form will predominate (95% in human plasma), and, thus, it is unlikely that GLUT-mediated DHA uptake will be sufficient for the cellular needs of most cells (Rose 1988, Dhariwal *et al.* 1991). Moreover, circulating levels of glucose are 1000-fold higher than the levels of DHA (~2–5 μM), and, thus, marked competition by glucose of DHA influx will occur. An exception may be in how vitamin C accumulates in the central nervous system. DHA formed by oxidation of ascorbic acid in the tissue microenvironment might lead to locally high concentrations of DHA that cross the blood–brain barrier via GLUT transporters (Agus *et al.* 1997). Once in the brain, DHA is reduced by an unknown mechanism and trapped as ascorbic acid.

Uptake of ascorbic acid by cells

Using radioactively labelled ascorbate, an independent concentrative uptake system for the reduced ascorbate has been demonstrated in guinea pig intestinal mucosal cells, human small intestinal brush-border membrane vesicles, rat osteoblasts, human neutophils and fibroblasts, 3T6 mouse fibroblasts, bovine cultured pigment cilary epithelial cells, astrocytes, human granulosa-leutin cells and cultured cell lines such as JAR human choriocarcinoma cells (Siliprandi *et al.* 1979, Padh and Aleo 1987, Helbig *et al.* 1989, Wilson and Dixon 1989, Wilson *et al.* 1991, Goldenberg and Schweinzer 1994, Welch *et al.* 1995, Prasad *et al.* 1998, Malo and Wilson 2000, Zreik *et al.* 1999). Influx was Na^+-dependent and kinetic characterization of the process revealed a high affinity for ascorbic acid with K_m values ranging from 10–200 μM (Padh and Aleo 1987, Helbig *et al.* 1989, Wilson and Dixon 1989, Goldenberg and Schweinzer 1994, Welch *et al.* 1995, Prasad *et al.* 1998, Zreik *et al.* 1999, Malo and Wilson 2000). In some cell types, JAR cells and 3T6 fibroblasts, sodium was shown to increase the affinity of ascorbic acid for the transporter without an effect on the maximum velocity (Padh and Aleo 1987, Prasad *et al.* 1998). However, in rat osteoblasts decreasing the external Na^+ concentration lowered both the affinity and the maximum velocity of transport (Wilson and Dixon 1989). In studies where it has been examined, the dependence of ascorbic acid uptake on the concentration of Na^+ exhibited sigmoidal kinetics with a Hill coefficient of 2 suggesting a Na^+ : ascorbate stoichiometry of 2 : 1 (Helbig *et al.* 1989, Wilson *et al.* 1991, Prasad *et al.* 1998). This stoichiometry implies electrogenicity of the transporter and addition of ascorbic acid to cultured ciliary epithelial cells resulted in a depolarization (Helbig *et al.* 1989). In epithelial cells (intestine and kidney), the Na^+-dependent transport system was found to be present at the brush-border membrane surface (Toggenburger *et al.* 1981, Bianchi *et al.* 1986, Rose 1986, Malo and Wilson 2000).

Molecular cloning of ascorbic acid transporters

In the absence of high-affinity inhibitors or antibody and oligonucleotide probes expression cloning in isolated oocytes of *Xenopus laevis* has become the strategy of choice to clone genes encoding transport proteins, for example Na^+-glucose and Na^+-nucleoside cotransporters (Hediger *et al.* 1987, Griffith and Jarvis 1996). The feasibility of this approach for ascorbic acid transporters was first established in 1994 by injecting rabbit renal poly(A)$^+$ mRNA into oocytes (Dyer *et al.* 1994). Screening of a rat kidney cDNA library for Na^+-dependent ascorbic acid transport activity identified a cDNA encoding a 604-amino acid protein, termed rSVCT1 (Tsukaguchi *et al.* 1999). Subsequent PCR-based homology screening identified a second related cDNA coding for a 592-amino acid protein, rSVCT2, with 65% identity to rSVCT1 (Tsukaguchi *et al.* 1999). Both rSVCT1 and rSVCT2 were shown to mediate concentrative, high-affinity L-ascorbic acid transport that is stereospecific and driven by the Na^+ electrochemical gradient.

Database analysis with rSVCT1 revealed that it shared 87% identity with YSPL3, a protein of unknown function but that had been designated a putative nucleobase transporter (Faaland *et al.* 1998). The high degree of similarity of YSPL3 to rSVCT1 suggested that it might be the human homologue of SVCT1. Isolation of the putative hSVCT1 transcript in our laboratory was performed using a series of PCR reactions and a human liver cDNA library. The human liver cDNA sequence was 1867 bp long with an open reading frame of 1797 bp encoding a 598-amino acid protein with a relative molecular mass of 64 823 (figure 1). A comparison of the DNA sequence of the hSVCT1 with that of YSPL3 revealed 26 codon discrepancies. Similar discrepancies were also noted by Daruwala *et al.* (1999) between their hSVCT1 sequence and YSPL3. However, there are no sequence differences between the hSVCT1 clones (figure 1, Daruwala *et al.* 1999, Wang *et al.* 1999, 2000) and the gene (*SLC23A2*) encoding hSVCT1 was mapped to the long arm of chromosome 5 in band 5q31.2–31.3. A non-functional splice variant of SVCT1 has been found in human intestine and Caco 2 cells with a four amino acid insert in the second extracellular loop between the putative transmembrane domains 3 and 4 (Wang *et al.* 1999). It remains to be determined whether YSPL3 encodes a functional ascorbic acid transporter.

At the same time as the above work on expression cloning of rat ascorbic acid transporters was being conducted, the laboratory was attempting to clone human nucleobase transporters based on homology to *Aspergillus nidulans* purine transporters. A full length clone (KF), derived from a human placental cDNA library, was obtained with an open reading frame of 1953 bp encoding a protein of 650 amino acids with a predicted mass of 70.4 kDa (figure 1). Nevertheless, when KF was transiently expressed in COS-1 cells it failed to enhance the uptake of hypoxanthine. However, and very surprisingly, the clone showed considerable similarity at the amino acid level to rSVCT1 (61% identity) and rSVCT2 (95% identity) strongly suggesting that the clone was the human homologue of SVCT2. Other workers using hSVCT1 to screen a JAR cell cDNA library, or RT-PCR with a human kidney cDNA library based on the YSPL2 sequence (a human clone of unknown function but 96% identity to rSVCT2) also obtained clones with identical open reading frames to the KF clone (Daruwala *et al.* 1999, Rajan *et al.* 1999, Wang *et al.* 2000). The clones have been designated hSVCT2 and *in situ* hybridization located the gene (*SLC23A1*) to human chromosome 20p13 (Hogue and Ling 1999).

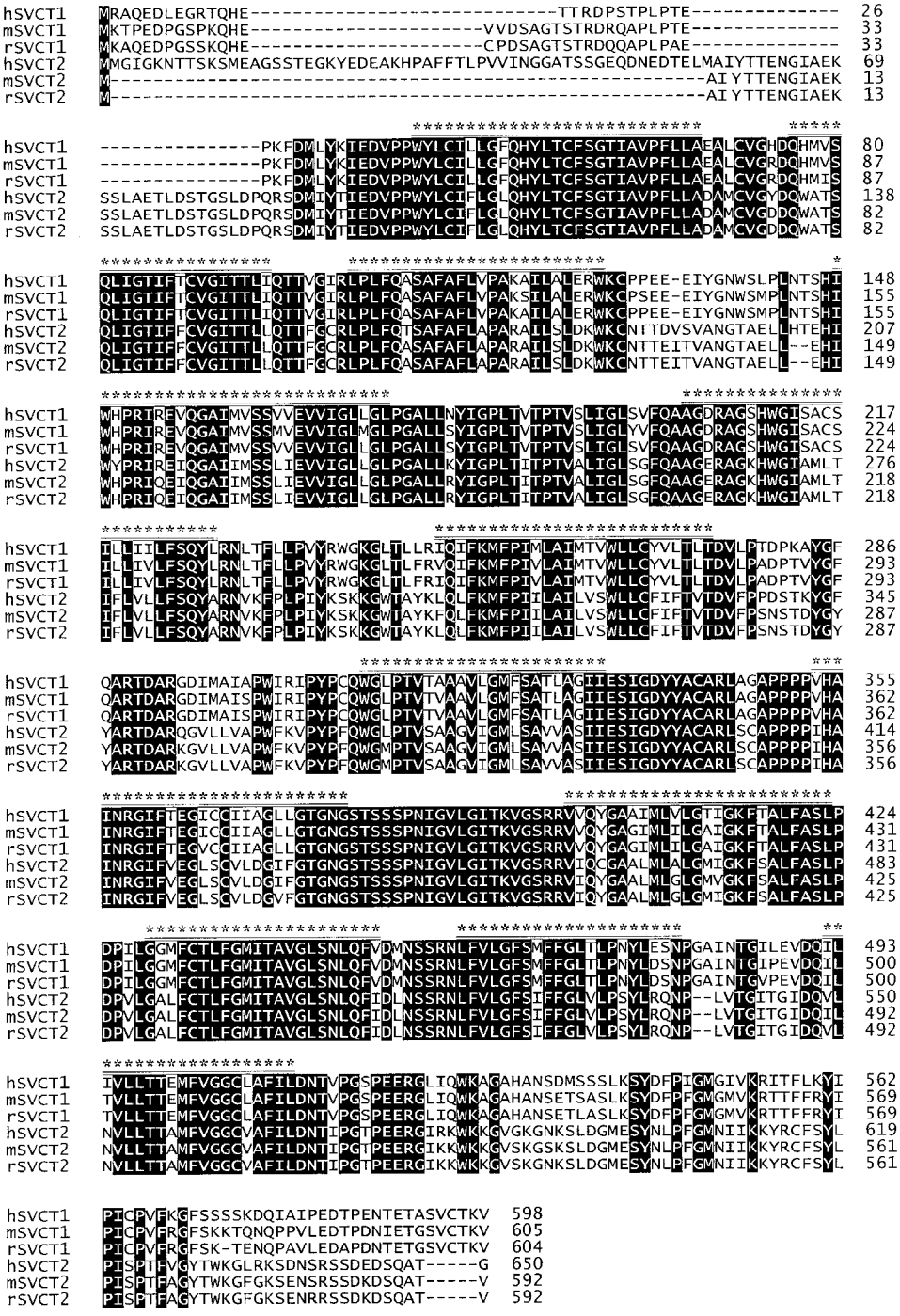

Figure 1. Alignment of the deduced amino acid sequences of the SVCT1 and SVCT2 proteins from human, mouse and rat. The amino acid sequences of hSVCT1 from human liver and hSVCT2 from human placenta were deduced from their respective cDNAs isolated by RACE-PCR (Accession No AJ250807 and AJ292318, respectively) and aligned with each other and the published sequence of rSVCT1 (AF080453), rSVCT2 (NM_017316), mSVCT1 (NM_018824) and mSVCT2 (AB038145). Gaps were introduced to optimize homology. Putative membrane spanning domains are indicated with *. Conserved residues are highlighted by shading.

Functional characterization of SVCT1 and SVCT2

To confirm that the above human clones were ascorbic acid transporters, the functional properties of either cDNA expressed in mammalian cell lines or cRNA injected into *Xenopus* oocytes have been studied. In our laboratory, the transient transfection system was used with COS-1 cells. A representative time course of [^{14}C]ascorbic acid uptake by COS-1 cells transfected with plasmids containing hSVCT1 in the correct and reverse orientation of the pCMV promoter are shown in figure 2. Ascorbic acid uptake was 14-fold higher in cells transfected with pcDNA3.1-hSVCT1 in the correct

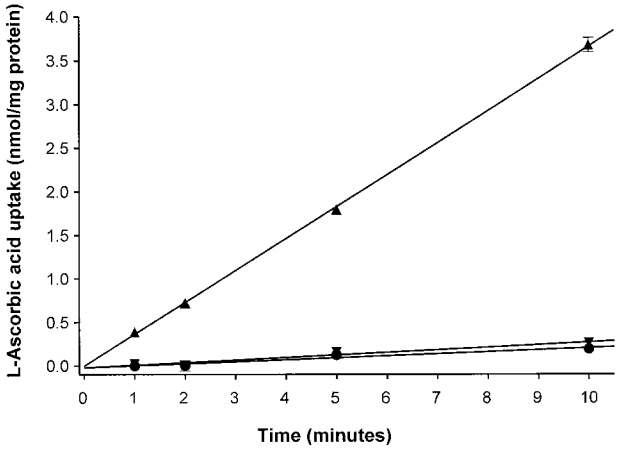

Figure 2. Time course of ascorbic acid uptake by recombinant hSVCT1 in transiently transfected COS-1 cells. Cells were transfected with pcDNA3.1-hSVCT1 in the correct (▲ and ●) or reverse orientation (▼) and assayed for ascorbic acid uptake 30 h after transfection. Ascorbic acid uptake at 22°C was initiated by the addition of transport buffer (0.3 ml) containing 200 μM [^{14}C]ascorbic acid in the presence (●) or absence (▲ and ▼) of 1 mM ascorbic acid. Values are the average ±SD of triplicate estimates. In subsequent experiments, an incubation time of 10–30 min was chosen to determine initial rates of ascorbic acid influx. hSVCT1/2-specific ascorbic acid transport was defined as the difference in transport rates between cells transfected with pcDNA3.1-hSVCT1 in the correct and reverse orientations or between transfected and non-transfected cells.

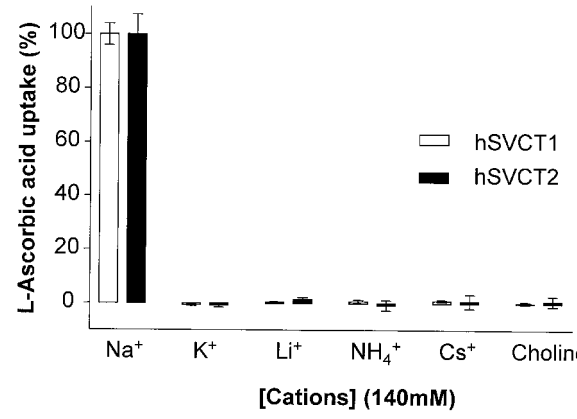

Figure 3. Effect of cations on ascorbic acid influx mediated by recombinant hSVCT1 and hSVCT2. The initial rate of 5 μM ascorbic acid uptake at 22°C by COS-1 transfected cells was determined in the presence of various monovalent cations as their chloride salts (140 mM). Uptake measured in control cells transfected with pcDNA3.1-hSVCT1 in the reverse orientation was similar to that of non-transfected cells and was subtracted from the test cells to obtain hSVCT1- (open bars) and hSVCT2- (solid bars) specific mediated uptake. Data expressed as a percentage of the flux in the presence of Na^+ and represent means ±S.E. of three independent determinations.

Table 1. Comparison of the kinetic constants of ascorbic acid uptake mediated by hSVCT1 and hSVCT2.

Expression system	Parameter	hSVCT1	hSVCT2
COS-1	K_m (μM)	78±19	28±6
Oocytes	K_m (μM)	90±17	58±12
		239–252	21–22
HRPE cells, 37°C	K_m (μM)	74±6	69±5
COS-1	V_{max} (pmol/mg protein)	860±360	260±15
Oocytes	V_{max} (pmol/min/oocyte)	15.5±1.2	1.2±0.1
		15.8	0.04
HRPE cells, 37°C	V_{max} (pmol/10^6 cells per 30 min)	No value reported	18.7±0.6

Kinetic parameters for cDNA-specific or cRNA-specific hSVCT1 and hSVCT2 transport have been taken from previously published work for expression using oocytes or the vaccina virus system with HRPE cells (Daruwala *et al.* 1999, Rajan *et al.* 1999, Wang *et al.* 1999, 2000).

orientation than in cells transfected in the reverse orientation indicating the production of functional, recombinant hSVCT1. Similar results were obtained with hSVCT2 transfected cells.

Uptake of ascorbic acid via both hSVCT1 and hSVCT2 is absolutely dependent on the presence of Na^+. Replacement of extracellular Na^+ with K^+, Li^+, $NH4^+$, Cs^+, or choline completely abolished ascorbic acid influx in hSVCT1- and hSVCT2-transfected COS-1 cells (figure 3). Limited studies in other expression systems have also shown that replacement of Na^+ with either choline or Li^+ resulted in >95% inhibition of ascorbic acid influx confirming the strict selectivity of Na^+ in driving ascorbic acid hSVCT1- and hSVCT2-mediated transport (Daruwala *et al.* 1999, Rajan *et al.* 1999, Wang *et al.* 1999, 2000). Varying the concentration of Na^+ at a fixed concentration of ascorbic acid (5 μM) revealed a sigmoidal dependence for both hSVCT1- and hSVCT2-mediated transport. Non-linear regression analysis of the data using the Hill equation yielded K_{Na} values (concentration of Na^+ giving 0.5 maximum flux) of 47±7 and 63±10 mM with Hill coefficients of 1.9±0.5 and 2.0±0.3 for hSVCT1 and hSVCT2, respectively. These results suggested that at least two Na^+ ions are transported per ascorbic acid molecule for both hSVCT1 and hSVCT2.

A comparison of the kinetic constants of the heterologously expressed transporters suggests that SVCT2 has a higher affinity for ascorbic acid than SVCT1 (table 1). The magnitude of the difference in K_m values varies between studies and ranges from 2 to10-fold. Using HRPE cells, no significant difference in K_m values at 37°C was observed between hSVCT1- and hSVCT2-mediated ascorbate uptake. (Rajan *et al.* 1999, Wang *et al.* 1999). This lack of difference may reflect the expression system used, as the K_m value determined with hSVCT2 cDNA from JAR cells expressed in HRPE cells using the vaccina virus system was 3-fold higher than the value obtained for ascorbic acid influx in JAR cells (Prasad *et al.* 1998, Rajan *et al.* 1999). A consistent finding in all the heterologous expression studies to date is that the V_{max} value for hSVCT1-mediated ascorbate influx is higher than that observed for hSVCT2-mediated ascorbate influx (table 1), suggesting that either less functional protein is inserted into the membrane or there are marked differences in the turnover number of the two carriers. Similarly, the currents evoked by rSVCT2 in oocytes were relatively small compared to rSVCT1 (Tsukaguchi *et al.* 1999), indicating that this difference between SVCT1 and SVCT2 is species independent.

The specificity of SVCT1 and SVCT2 has been mainly investigated by examining the effects of various compounds to inhibit ascorbic acid influx. Both transporters exhibit a strict stereospecificity with the stereoisomer of L-ascorbic acid, D-isoascorbic acid, ~30-fold less potent than L-ascorbic acid at inhibiting [^{14}C]ascorbic acid influx in transfected COS-1 cells (mean IC_{50} values of 2400 ± 490 and 700 ± 100 μM ($n = 3$) for hSVCT1 and hSVCT2, respectively). D-isoascorbic acid at high concentrations evoked a small current, 15–30% of that of L-ascorbic acid, in oocytes expressing hSVCT1 and rSVCT1, confirming that D-isoascorbic acid is a low affinity permeant for the transporter (Tsukaguchi *et al.* 1999, Wang *et al.* 2000). Dehydroascorbic acid, glucose, deoxyglucose, xanthine, hypoxanthine, ascorbic acid 2-phosphate, ascorbic acid 2-sulphate and L-gulono-γ-lactone had no effect on hSVCT1- and hSVCT2-mediated ascorbic acid uptake in COS-1 cells. A range of nucleobases, nucleosides, nucleotides and vitamins failed to inhibit ascorbic acid uptake in oocytes expressing hSVCT1 and HRPE cells transfected with hSVCT1 and hSVCT2 (Rajan *et al.* 1999, Wang *et al.* 1999, 2000).

The effect of extracellular pH on the uptake of ascorbic acid in hSVCT1- and hSVCT2-transfected COS-1 cells has been compared (figure 4). The optimum pH for transport in both cases was ~pH 7.5 and was reduced by ~90% at pH 5.5. A decrease in transport activity was also observed as the pH increased above the optimum for hSVCT2, but this was not observed for hSVCT1-mediated transport. At physiological pH, ascorbic acid exists as a monovalent anion and, thus, if two Na^+ ions are transported per ascorbic acid molecule as suggested from the Na^+ activation curves, the transport process will be electrogenic and respond to both changes in the Na^+ gradient as well as membrane differences. The decrease in ascorbic acid uptake at more acid pH is unlikely to be due to protonation of the permeant as the pKa for ascorbic acid is 4.2 and more than 95% of L-ascorbic acid is in the -1 form at pH 5.5. Rather, the decrease in transport activity at acid pH is likely to be the result of reduced binding affinity. Indeed, preliminary studies from this laboratory have shown that at pH 6.5, the K_m value increased as compared to the value at pH 7.5, with no significant change in V_{max} (K_m values of 340 ± 43 and 200 ± 32 μM for hSVCT1 and 52 ± 6 and 25 ± 8 μM for hSVCT2 at pH 6.5 and 7.5, respectively). The pH dependency profile from pH 5.5–7.4 for both hSVCT1 and hSVCT2 is similar (figure 4) and possibly suggests the involvement of histidine. There are four conserved histidine residues in the proteins (figure 1).

Proposed membrane topology of SVCT1 and SVCT2

Hydropathy plots of the amino acid sequence of SVCT1 and SVCT2 are consistent with 12 potential transmembrane (TM) domains (see figure 5). A putative model for hSVCT1 and hSVCT2 based on the hydrophobicity plots, the 'positive inside' rule and a comparison of all the known SVCT sequences is shown in figure 5. Both proteins are predicted to contain 12 TM segments, with the N and C termini on the cytoplasmic side of the membrane. The N-termini of hSVCT1 and hSVCT2 contain a high proportion of acidic residues (>20%), properties consistent with this region acting as a potential regulatory domain that may interact with other proteins. Also, a comparison of hSVCT1 with rSVCT1 and mSVCT1 reveals the absence of seven amino acid residues in the amino terminus. Two potential N-glycosylation sites are present in both hSVCT1 and hSVCT2 between TM3 and 4, with an additional site between TM 5 and 6 that is only present in hSVCT1. Comparison of the translation products of hSVCT1 and hSVCT2 synthesized *in vitro* revealed lower molecular weight proteins in the absence of microsomes, suggesting that hSVCT1 and hSVCT2 are glycosylated in vivo (Wang *et al.* 2000).

An obvious difference between hSVCT1 and hSVCT2 is the additional sequences of 12 and 44 amino acids that are present in the N terminal end of hSVCT2 at positions 2 and 38, respectively. There are also five potential sites for protein kinase C-dependent phosphorylation located on the cytoplasmic surface for both proteins, two of which are located in the C terminus. An additional protein kinase A site is present in the C-terminal of hSVCT1 that is not found in

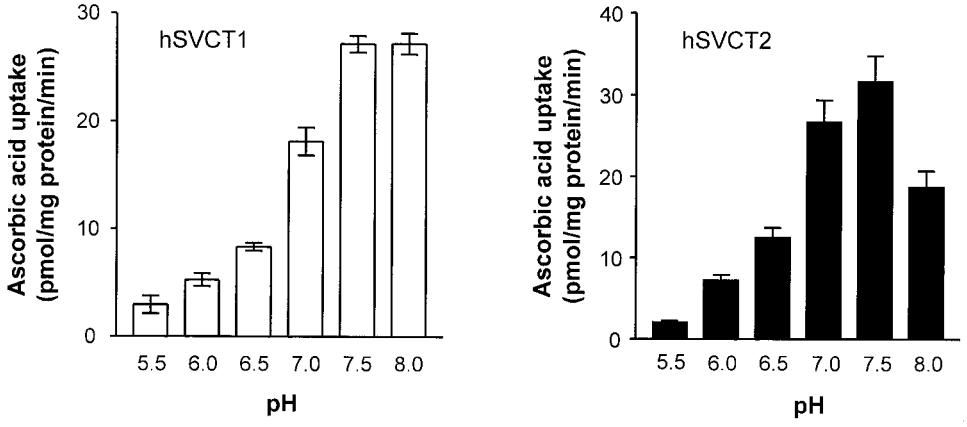

Figure 4. Effect of extracellular pH on hSVCT1- and hSVCT2-mediated ascorbic acid influx in transfected COS-1 cells. The initial rate of 5 μM ascorbic acid uptake at 22°C was determined in the presence of varying pH and specific-mediated ascorbic acid plotted as described in the legend to figure 3.

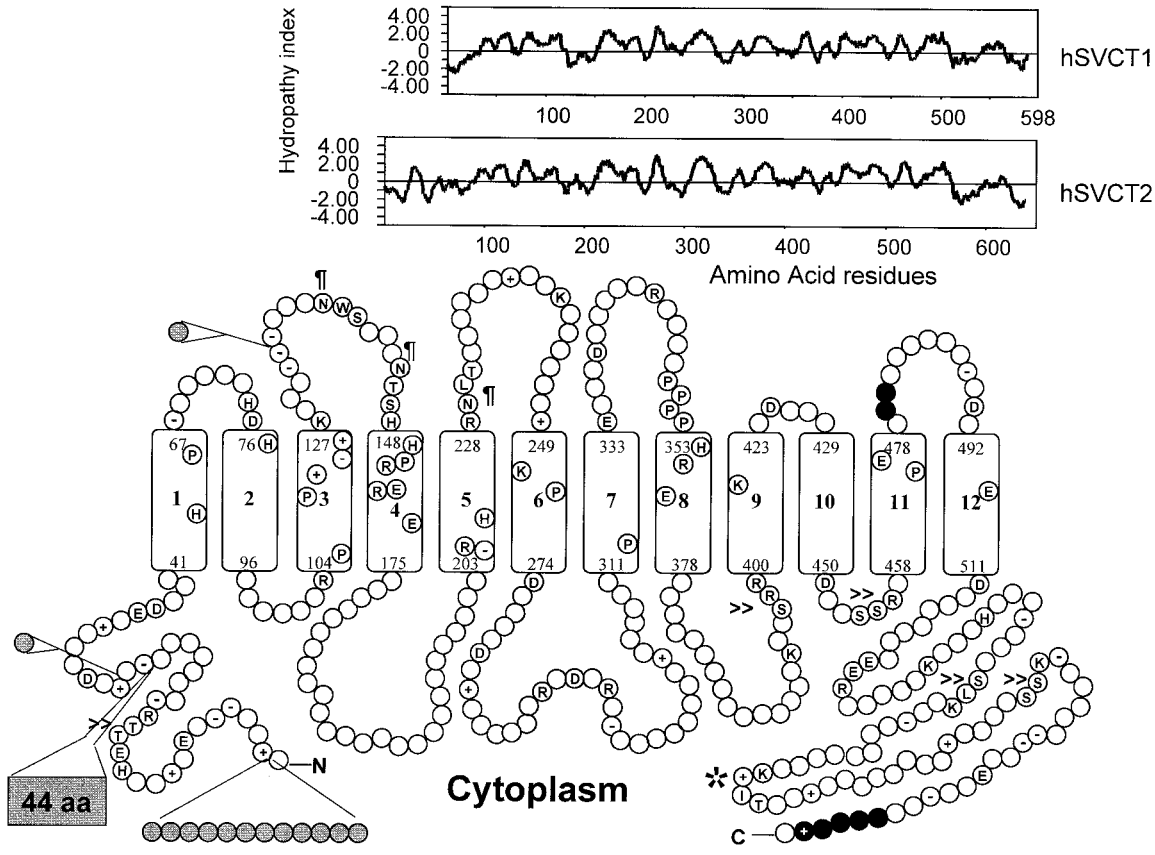

Figure 5. Hydropathy analyses and topographical model of hSVCT1 and hSVCT2. Hydropathy profile for the predicted amino acid sequence of hSVCT1 and hSVCT2 was determined by the method of Kyte-Doolittle with a widow size of 11. Potential membrane spanning domains are numbered and the consensus sites for *N*-linked glycosylation (¶), and protein kinase C (>>) and A (★) phosphorylation are shown. The distributions of certain amino acid residues are also shown using either the single letter code or + and — to represent positively and negatively charged amino acids, respectively. Amino acid residues that are absent from hSVCT2 are indicated as (●), whereas those residues that are only present in hSVCT2 are shaded.

hSVCT2 and is suggestive of differential regulation of the two carriers. Cyclic AMP has been demonstrated to stimulate the uptake of ascorbic acid in rat astrocytes after a latent period of 12 h, and stimulation was blocked by cycloheximide suggesting a requirement for *de novo* protein synthesis (Siushansian *et al.* 1997). Activation of protein kinase C has been shown to inhibit Na^+-dependent ascorbate transport in a cell line derived from rabbit non-pigmented ciliary epithelium and in oocytes expressing hSVCT1 and hSVCT2 (Delamere *et al.* 1993, Daruwala *et al.* 1999). The mechanism by which the protein kinase C stimulator, PMA, acts is unknown.

Helical wheel analyses of the putative TM segments in SVCT1 and SVCT2 revealed that TM 1, 4, 5 and 8 are amphiphilic. TMs 3, 6, 9 and 12 in hSVCT1 are hydrophobic, as defined by possessing less than four charged residues per 18 amino acids, and may act as stabilizing regions for the transporter as proposed for the lactose permease (Zhuang *et al.* 1999). Four charged residues within the putative TM regions of SVCT1 and SVCT2 are not conserved (residues 209, 370 476 and 500 in TM 5, 8, 11, 12 of hSVCT1, respectively), with the net effect being to make the internal environment of SVCT2 more positive than SVCT1.

A unique structural feature in all the SVCTs is a string of conserved proline residues (Pro 349–352 in hSVCT1) located in the extracellular loop 7 between TM 7 and 8. These proline residues may play an important role in keeping the putative TM helices 7 and 8 in place for transport function and possibly suggests that TM 7 and 8 are involved in the substrate binding and subsequent translocation of ascorbic acid. Seven additional proline residues are also predicted to lie within the TM segments (figure 5) and these are conserved amongst SVCT1 and SVCT2 proteins, suggesting they may also play an important role in determining the structure of ascorbic acid transporters.

Tissue distribution of SVCT1 and SVCT2

The tissue distribution of SVCT2 as analysed by Northern blots was much wider than that of SVCT1. For hSVCT2, a 7.5 kb transcript was detected in most tissues tested, with the notable exception of lung and skeletal muscle (Rajan *et al.* 1999, Wang *et al.* 2000). Probing for hSVCT1 resulted in a strong signal at ~2.4–3 kb in kidney, liver, small intestine, colon, ovary and prostate (Wang *et al.* 1999, 2000). A transcript of ~9 kb was detected in thymus that may result

from alternative splicing or correspond to a closely related gene product distinct from SVCT2. *In situ* hybridization with rSVCT1 and rSVCT2 confirmed the above distributions and further localized rSVCT1 to the straight segment (S3) of the proximal tubule in the kidney (Tsukaguchi *et al.* 1999). The differential tissue expression of SVCT1, largely confined to epithelial tissues, compared to the widespread location of SVCT2, and the kinetic properties of SVCT1 (low affinity, high V_{max}) supports the hypothesis that the two transporters are polarized within the cell, with SVCT1 targeted to brush-border surfaces of epithelial cells and SVCT2 to the basolateral surface. Immunocytochemistry studies will be required to confirm this speculation.

Phylogenic analysis

Analysis of homologous sequences that align with hSVCT1 in the form of a cladogram is shown in figure 6. SVCT belong to a novel family of membrane transporters that do not share structural homology with other families of Na^+-cotransporters. Figure 6 demonstrates that of the 14 most closely related sequences to hSVCT1, SVCT1 and SVCT2 have a common ancestor that diverged before rodents and mammals. Calibrating the molecular clock by taking the divergence of mice and rats at 20–29 million years ago (O'hUigin and Li 1992), the date of divergence of SVCT1 and SVCT2 can be estimated to have occurred between 280–400 million years ago. It is interesting to note that the emergence of specific expression of L-glucone-γ-lactone oxidase, a key ascorbate biosynthetic enzyme, has been correlated with the emergence of terrestrial vertebrates which occurred ~360 million years ago (Nandi *et al.* 1997). Hence, the divergence of SVCT1 and SVCT2 may be linked to the tissue specific expression of L-glucone-γ-lactone oxidase, which in turn has been postulated to have been stimulated by the exposure of emergent terrestrial vertebrates to an oxygen-rich environment and the need to develop free radical scavengers (Nandi *et al.* 1997). The *Aspergillus nidulans* UapA and UapC purine/pyrimidine transporters are only distantly related to the ascorbic acid transporters, although a signature sequence defined for UapA and UapC (Prosite ID: PDOC00860) is also found in the ascorbic acid transporters.

Conclusion

In conclusion, the cloning of SVCTs in the last year should lead to the further characterization of structural features responsible for transport and to studies on the regulation of ascorbic acid transport in both physiological and pathological conditions. The cloning work also opens the possibility of identifying additional isoforms of SVCT. Increasing knowledge of the ascorbic acid transporters should facilitate one's understanding of the role in vitamin C transport in chronic disease prevention.

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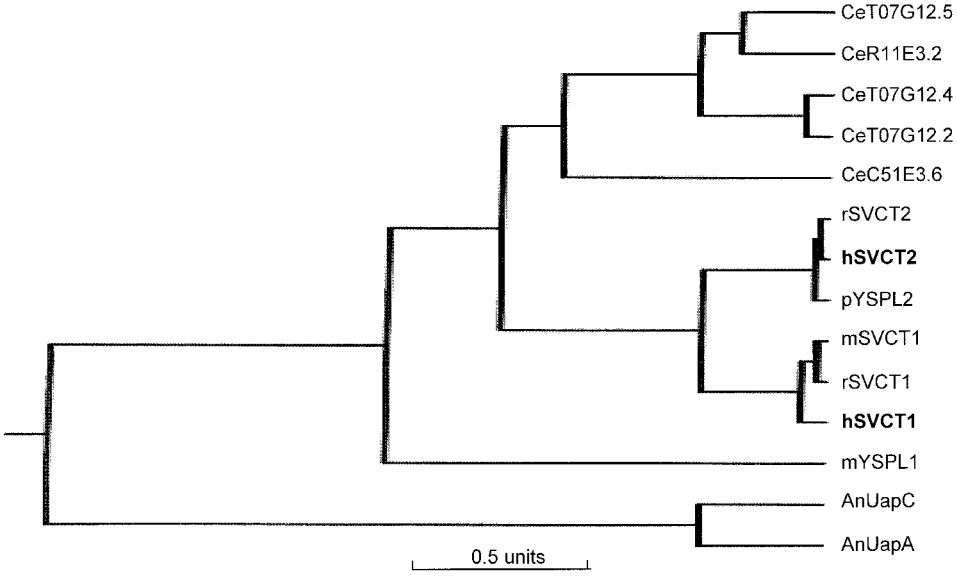

Figure 6. Cladogram of the vitamin C transporters. The KITSCH program of the PHYLIP (Felsenstein 1996) package was used to construct the cladogram, using the Dayhoff PAM matrix. The sequences were bootstrapped with 25 replicates. All the branch interstices of the cladogram above were produced 25 times, with the exception of the interstice between rSVCT2 and hSVCT2 which was found 18 times. The branch lengths are proportional to time. The branch length between mSVCT1 and rSVCT1 is 0.0184 units and the branch length of divergence of the two groups of vitamin C transporters is 0.256 units. Taking the time of divergence of mice and rats 20–29 million years ago (O'hUigin and Li 1992), the time of divergence of the two vitamin C transporters is estimated to be 270–400 million years ago. Abbreviations used: r; rat, h; human, m; mouse, p; pig, Ce; *Caenorhabditis elegans*, An; *Aspergillus nidulans*. The Genbank accession numbers are: AnUapA (X71807), AnUapC (X79796), CeC51E3.6 (Z78410), CeR11E3.2 (AF100669), CeT07G12.2 (Z82282), CeT07G12.4 (Z82282), CeT07G12.5 (Z82282), hSVCT1 (AJ250807), hSVCT2 (AJ292318), mYSPL1 (U25739), mSVCT1 (NM_017316), pYSPL2 (AF058320), rSVCT1 (AF080453), rSVCT2 (NM_017316).

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