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Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2

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Abstract Two sodium-dependent vitamin C transporters, hSVCT1 and hSVCT2, were cloned from a human kidney cDNA library. hSVCT1 had a 1797 bp open reading frame encoding a 598 amino acid polypeptide. The 1953 bp open reading frame of hSVCT2 encoded a 650 amino acid polypeptide. Using a *Xenopus laevis* oocyte expression system, both transporters were functionally expressed. By Eadie-Hofstee transformation the apparent K_m of hSVCT1 for ascorbate was 252.0 μM and of hSVCT2 for ascorbate was 21.3 μM. Both transporters were sodium-dependent and did not transport dehydroascorbic acid. Incubation of oocytes expressing either transporter with phorbol 12-myristate 13-acetate (PMA) inhibited ascorbate transport activity. Availability of the human transporter clones may facilitate new strategies for determining vitamin C intake.

Key words: Vitamin C; Ascorbate; Transport; hSVCT1; hSVCT2

1. Introduction

Ascorbic acid (ascorbate, vitamin C) is essential for human health. Most mammals synthesize ascorbate, but human and non-human primates are unable to synthesize and must ingest ascorbate [1,2]. The known enzymatic roles of vitamin C include collagen hydroxylation, carnitine biosynthesis, and formation of the catecholamine norepinephrine [2,3]. Other studies have emphasized the role of ascorbic acid as a chemical electron donor or antioxidant [4,5].

Ascorbate accumulation in tissues occurs by two general pathways [6]. In one pathway, ascorbate is oxidized extracellularly to dehydroascorbic acid and transported via glucose transporter isoforms GLUT1 and GLUT3 [6,7]. Once internalized dehydroascorbic acid is rapidly reduced by glutaredoxin and other proteins to ascorbate [8–11]. The dehydroascorbic acid pathway is dependent on substrate formation, as the substrate is not always present. In another distinct pathway, ascorbate is transported as such in a sodium-dependent manner [6,12]. In humans the ascorbate pathway is dependent on substrate availability via diet.

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Abbreviations: BLAST, basic local alignment search tool; GLUT, glucose transporter; hSVCT, human sodium-dependent vitamin C transporter; PCR, polymerase chain reaction; rSVCT, rat sodium-dependent vitamin C transporter; SMART, simple modular architectural research tool; YSPL, yolk sac permease-like molecule or nucleobase transporter

In rats two sodium-dependent ascorbate transport proteins, rSVCT1 and rSVCT2, were recently cloned [13]. Humans, unlike rats, cannot make ascorbate, and the characterization of the human transporter is therefore necessary and could have implications for dietary recommendations. In this paper we have cloned and expressed two human ascorbate transporters, hSVCT1 and hSVCT2.

2. Materials and methods

2.1. Cloning and sequencing of human transporters hSVCT1 and hSVCT2

Full length human hSVCT1 and hSVCT2 clones were generated by polymerase chain reaction (PCR) screening of a Marathon Ready kidney cDNA library (Clontech) [14]. PCR products were purified by agarose gel electrophoresis and cloned into pGEM-T Easy vectors (Promega). Double strand sequence analysis of three hSVCT1 and three hSVCT2 clones was performed to rule out ambiguities or PCR-introduced mutations.

2.2. cRNA synthesis of hSVCT1 and hSVCT2

hSVCT1 and hSVCT2 were linearized prior to in vitro transcription. Capped cRNA was synthesized using the mMessage mMachine kit (Ambion). The RNA integrity, size and concentration were measured by agarose gel electrophoresis.

2.3. Oocyte isolation and injection

Oocytes were isolated from *Xenopus laevis* sexually mature females [7,15]. Isolated oocytes were injected using a Nanoject II injector (Drummond Scientific). Injection volumes were 36 nl and cRNA concentrations 1 ng/nl. Sham oocytes were injected with 36 nl sterile water. Experiments were conducted 3 days post-injection.

2.4. Ascorbate transport

Ascorbate transport was measured as previously described for glucose transporters [7]. Briefly, 10–15 oocytes were incubated with [^{14}C]ascorbate (NEN Life Science Products, 8.0 mCi/mmol) at 23°C in OR-2 (5 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 100 μg/ml gentamicin, pH 7.8). Oocytes were then rapidly washed four times with ice-cold phosphate buffered saline and lysed in 200 μl 10% SDS. Internalized radioactivity was measured by scintillation spectroscopy. For transport in the absence of Na^+, equimolar choline chloride was used instead of NaCl. When used, dehydroascorbic acid was synthesized as described [6].

PMA, dibutyryl cyclic (dc) AMP and ascorbate were purchased from Sigma Chemical Co. Other reagents used were of the highest chemical grade.

2.5. Statistical and kinetic calculations

The data are expressed as arithmetic mean ± S.D. of 10–15 oocytes at each data point. Transport kinetics were calculated using Lineweaver-Burk and Eadie-Hofstee transformations.

3. Results

3.1. Cloning of the human vitamin C transporter hSVCT1

A BLAST (basic local alignment search tool) search with rSVCT1 revealed it shares 95% identity with the human nu-

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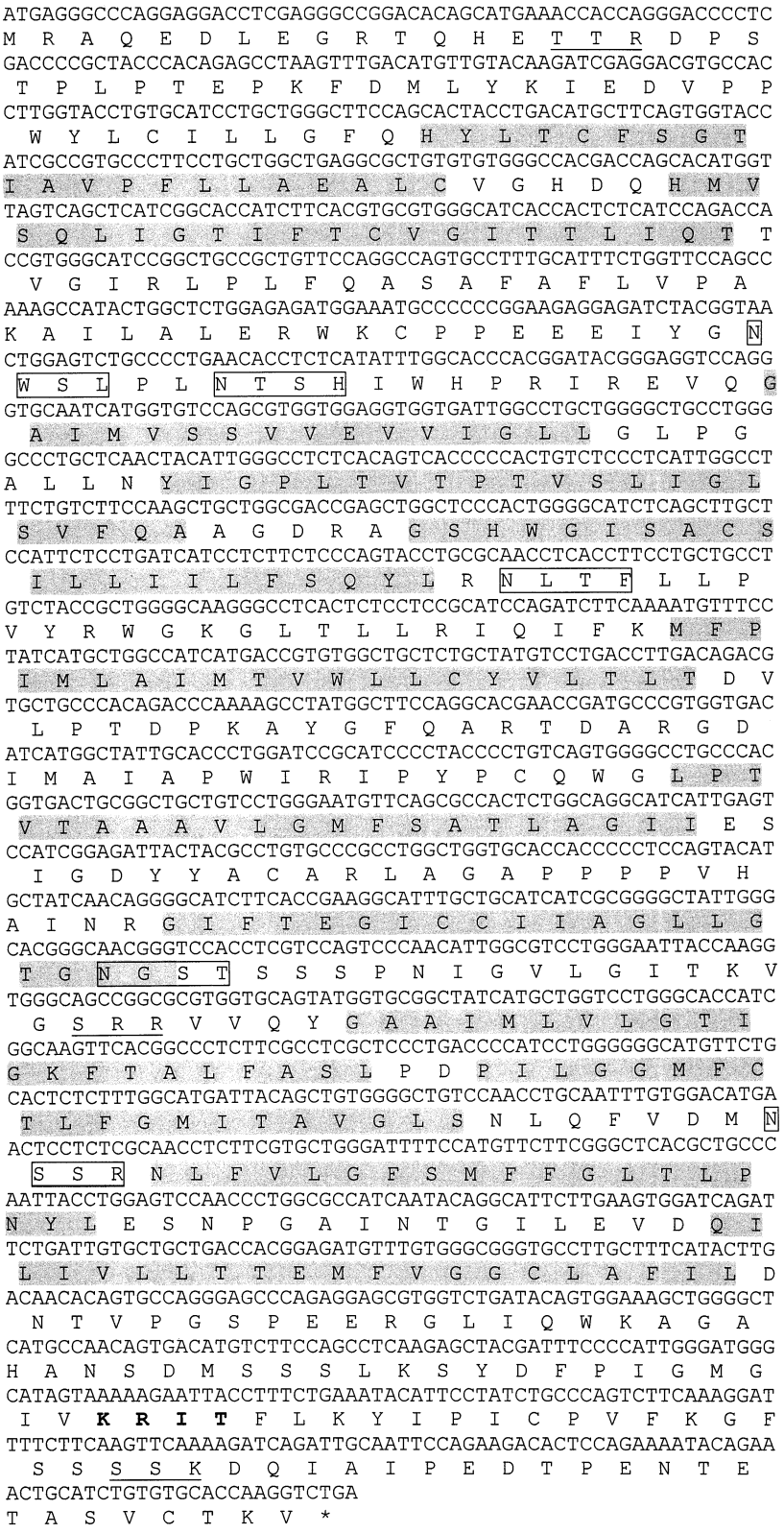

Fig. 1. Nucleotide sequence of human hSVCT1 with the predicted amino acid sequence. Shown are predicted transmembrane segments (gray shade), protein kinase C phosphorylation motifs (underlined), protein kinase A motif (bold), and ASN- glycosylation sites (boxed).

cleobase transporter 3 (YSPL3) [16]. YSPL3 was classified based on its 31% similarity to nucleobase transporter 1 (YSPL1).

Using PCR primers for YSPL3, a human kidney cDNA library was screened. An 1800 bp PCR product was cloned and three clones were sequenced. The sequences of the three clones were identical. Sequence analysis revealed a 1797 bp open reading frame, encoding a predicted 598 amino acid

polypeptide (Fig. 1). There were 41 nucleotide differences between the new clone and YSPL3. A simple modular architectural research tool (SMART) analysis of the predicted polypeptide identified 12 transmembrane segments consistent with transport proteins [17]. The new clone was termed hSVCT1 (accession number AJ 269477).

3.2. Cloning of the human vitamin C transporter hSVCT2

A BLAST search with rSVCT2 identified YSPL2 as a possible human homolog. YSPL2 was 96% identical to rSVCT2 but only 31% similar to YSPL1. Using PCR primers for YSPL2, a human kidney cDNA library was screened. A 2000 bp PCR product was cloned and three clones were sequenced. Sequence analysis identified a 1950 bp open reading frame, encoding a predicted 650 amino acid polypeptide. There was no sequence difference between the new clones and YSPL3. The clones were termed hSVCT2 (accession number AJ 269478).

3.3. Functional expression of hSVCT1 and hSVCT2 in X. laevis oocytes

To investigate the functional properties of hSVCT1, cRNA was injected into *X. laevis* oocytes. *Xenopus* oocytes lack endogenous ascorbate transporters and sham-injected oocytes did not accumulate detectable levels of [^{14}C]ascorbate (Fig. 2A,B). As seen in Fig. 2A, hSVCT1-injected oocytes exhibited a 100-fold increase in ascorbate transport. Transport was sodium-dependent, indicated by loss of transport in the presence of choline. Dehydroascorbic acid was not transported by hSVCT1.

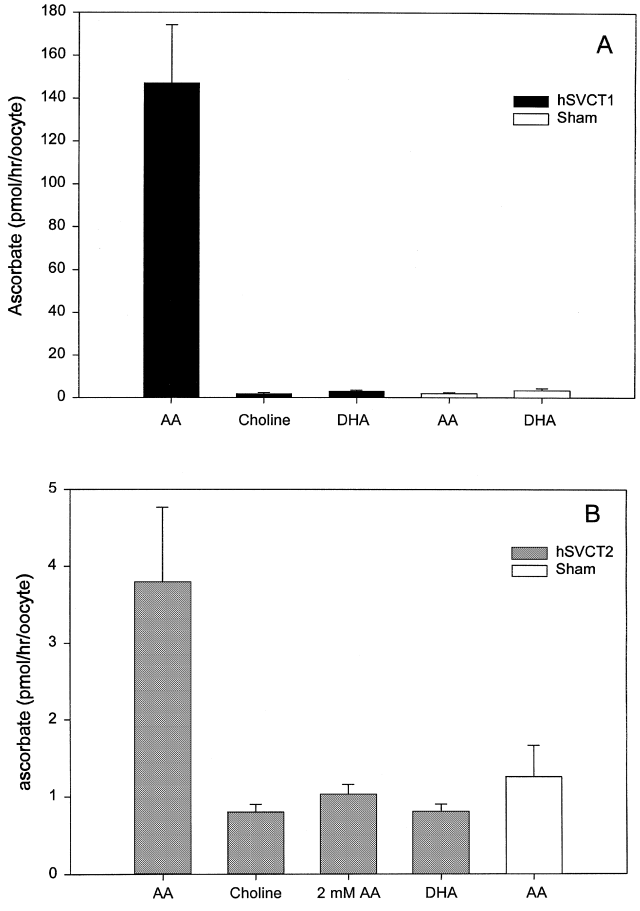

Fig. 2. Functional expression of hSVCT1 (A) and hSVCT2 (B) in *Xenopus* oocytes. Oocytes were incubated for 60 min with 100 μM [^{14}C]ascorbate (AA); choline and 100 μM [^{14}C]ascorbate; 2 mM unlabeled AA and 100 μM [^{14}C]ascorbate; 500 μM [^{14}C]dehydroascorbic acid (DHA). Sham-injected oocytes (□) were incubated with 100 μM [^{14}C]ascorbate or 500 μM [^{14}C]DHA.

Oocytes expressing hSVCT2 transporters also transported ascorbate in a sodium-dependent manner (Fig. 2B). Addition of excess unlabelled ascorbate inhibited [^{14}C]ascorbic acid accumulation, confirming that ascorbate accumulation was not due to diffusion. Similar to hSVCT1, hSVCT2 did not transport dehydroascorbic acid.

3.3. Ascorbate transport kinetics

To examine the kinetics of ascorbic acid transport, oocytes expressing hSVCT1 transporters were incubated with increasing concentrations of [^{14}C]ascorbate. Ascorbate uptake was saturable with an apparent K_m of 233.8 μM and V_{max} of 13.7 pmol/min/oocyte calculated by Lineweaver-Burk transformation. By Eadie-Hofstee transformation the K_m was 252.0 μM and V_{max} was 15.8 pmol/min/oocyte.

Oocytes expressing hSVCT2 were also incubated with increasing ascorbate concentrations. hSVCT2 transport conformed to simple Michaelis-Menten kinetics (Fig. 3B). The calculated apparent K_m for ascorbate from the double reciprocal plot was 22.6 μM, and V_{max} was 0.2 pmol/min/5 oocytes. The K_m by Eadie-Hofstee transformation was 21.3 μM and V_{max} was 0.2 pmol/min/5 oocytes.

3.4. Effect of PMA and dcAMP on hSVCT1 and hSVCT2

A SMART pattern analysis search of hSVCT1 and hSVCT2 identified five possible protein kinase C phosphorylation sites for hSVCT1 (Fig. 1), and six for hSVCT2 (not shown). Analysis also identified a probable cyclic AMP phosphorylation motif in hSVCT1 (Fig. 1).

To test the possible significance of these motifs, oocytes expressing hSVCT1 were incubated with the protein kinase C stimulator PMA. This resulted in a 10-fold decrease in ascorbate accumulation (Table 1). The effect on PMA on hSVCT2 was also significant with a 50% decrease in ascorbate accumulation. These findings indicate both hSVCT1 and hSVCT2 were sensitive to phorbol 12-myristate 13-acetate.

The cyclic AMP analog dcAMP had no significant effect on ascorbate uptake in oocytes expressing either transporter.

4. Discussion

In this study we cloned and functionally expressed the human vitamin C transporters hSVCT1 and hSVCT2. Based on homology, hSVCT1 and hSVCT2 were identified as nucleobase transporters YSPL3 and YSPL2, respectively [14]. The sequence analysis of hSVCT1 identified differences between it and YSPL3. These differences may have resulted in a non-functional YSPL3 clone. Although the hSVCT2 and YSPL2 clones were identical, YSPL2 did not have a functional component [14].

The kinetic data concerning the K_m for hSVCT2 of 22.2 μM are consistent with plasma and tissue distribution of vitamin C and localization of SVCT2. The wide tissue distribution of rSVCT2 and YSPL2 implicates hSVCT2 as the cellular ascorbate transporter [13,14]. In humans, plasma concentration of vitamin C as a function of dose is tightly controlled. At daily

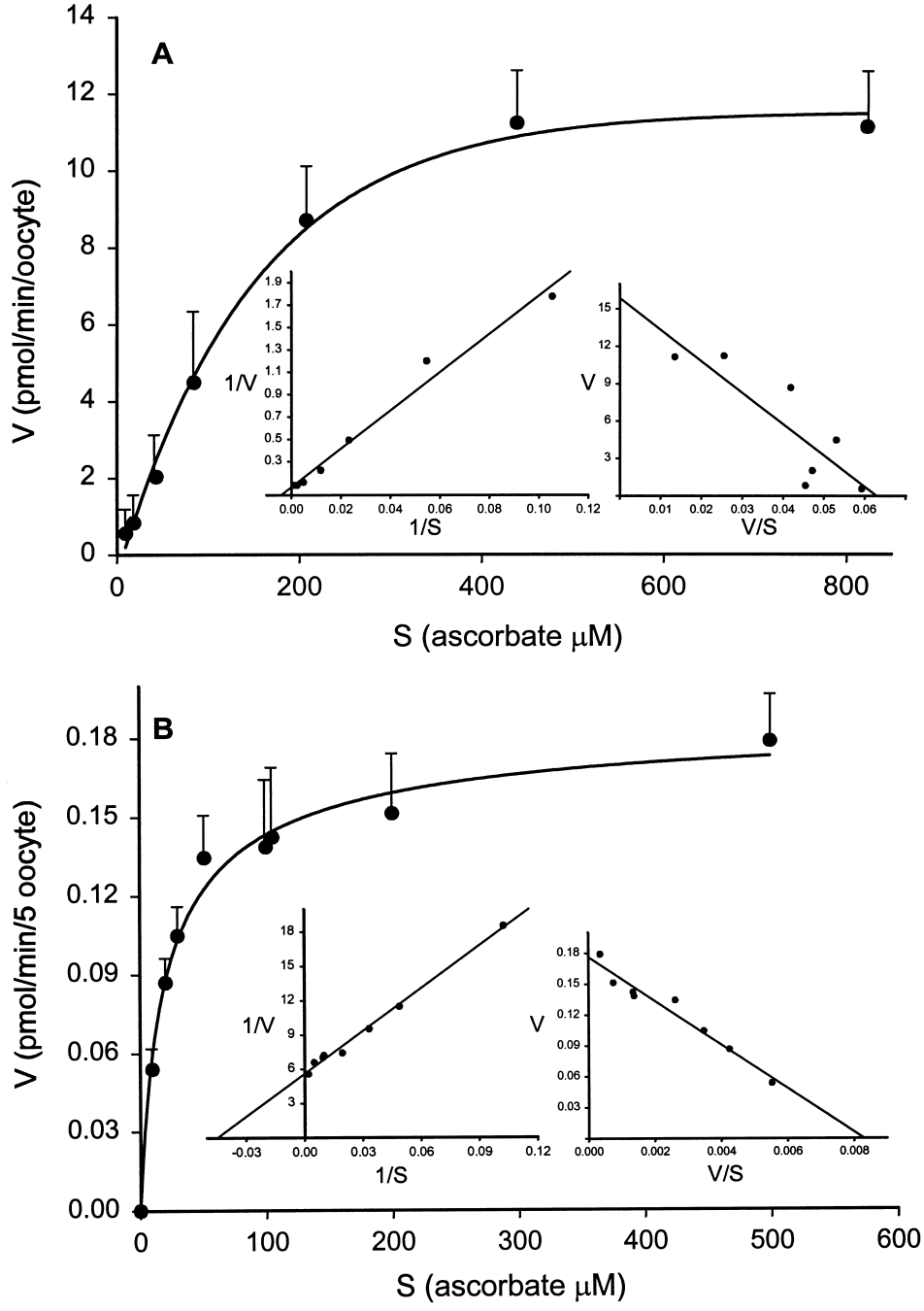

Fig. 3. Ascorbate concentration-dependent transport in *Xenopus* oocytes. Oocytes expressing hSVCT1 (A) or hSVCT2 (B) were incubated for 15 and 60 min, respectively, with 0–850 μM [^{14}C]ascorbate. Internalized radioactivity was measured as described in Section 2. Insets are Lineweaver-Burk and Eadie-Hofstee transformations of the data.

vitamin C doses of 200–250 mg, obtainable by eating five fruits and vegetables, plasma vitamin C concentration is 80% saturated and is approximately 65–70 μM [2,18]. Steady-state plasma vitamin C concentration probably does not exceed 100 μM despite ingestion of many grams of vitamin C [18,19]. At plasma concentrations of approximately 60 μM, tissues appear to be saturated with vitamin C [18]. A K_m of 22.2 μM for hSVCT2 is concordant with these observations and with observed sodium-dependent ascorbate transport in human cells [12].

The K_m for hSVCT1 of 237.3 μM was approximately 10-fold higher that than for hSVCT2. Explanations of these observations might be different tissue distributions of the two transporters and available vitamin C concentrations in the different tissues. Because Northern blot analyses of rSVCT1 and YSPL3 localized their distribution to the small intestine, kidney and liver [13,14], the primary role of hSVCT1 may be in intestinal absorption and renal tubular reabsorption of vitamin C. After ingestion of 100 mg of vitamin C, an amount readily obtained from a meal containing fruits and vegetables, it is possible that intraluminal intestinal concentrations of vitamin C could be 200 μM. Although the renal tubule con-

Table 1
Effect of included PMA and dcAMP on ascorbate transport

Conditions	hSVCT1 (pmol/h/oocyte)	hSVCT2 (pmol/h/5 oocytes)
None	198.2 ± 27.0	10.87 ± 0.3
PMA	17.5 ± 3.7	5.9 ± 1.6
dcAMP	141.9 ± 37.8	10.6 ± 1.8

Xenopus oocytes injected with hSVCT1 or hSVCT2 cRNA were incubated with PMA (100 nM) or dcAMP (100 μM) for 60 min and 100 μM [^{14}C]ascorbate. Ascorbate transport was measured as described in Section 2.

centration of vitamin C is unknown, it may be higher than the vitamin C plasma concentration due to reabsorption of sodium and water in the tubule before the site of vitamin C reabsorption. For these reasons, it can be predicted that the K_m of the vitamin C transporter in intestine and kidney will be higher than that of hSVCT2. Indirect evidence supporting the validity of the K_m calculation for hSVCT1 is that bioavailability of vitamin C is nearly complete at doses of 200 mg [18,19], when intraluminal intestinal vitamin C concentration could be as high as 0.5 mM.

Intestinal and tissue transport plays an essential role in developing recommendations for vitamin C intake [2]. With the availability of the human transporter clones, it may become possible in the near future to consider recommendations for vitamin C intake in relation to normal or aberrant transporter function. Intake guidelines might then account for individuals with decreased transporter number or functionally impaired transporters due to mutations or inappropriate regulation. For example, our preliminary studies of the possible role of the protein kinase C stimulator phorbol myristate acetate show that it inhibits hSVCT1 and hSVCT2 transport activity. These data may provide insight into the role of protein kinase C as a regulator of hSVCT1 and hSVCT2. Studies to consider variations in transport function and subsequent effect on vitamin absorption and distribution may have considerable implications for dietary recommendations.

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