Supplementary information relating to Nature, Vol. 399, pages 70-75, May 6, 1999

A family of mammalian Na^+-dependent L-ascorbic acid transporters

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Supplementary Information

Methods

Cloning of SVCT cDNAs. SVCT1 cDNA was isolated from rat kidney by expression cloning with heterologous expression in *Xenopus* oocytes as described[1]. Briefly, poly(A)$^+$ RNA was extracted from kidney cortex of normal male Sprague-Dawley rats. This poly(A)$^+$ RNA induced a 30-fold increase in Na^+-dependent L-[^{14}C]ascorbic acid uptake activity in oocytes compared with control (water-injected) oocytes (data not shown). Poly(A)$^+$ RNA was size-fractionated using preparative gel electrophoresis, and a positive poly(A)$^+$ RNA fraction inducing maximal L-[^{14}C]ascorbic acid uptake activity (1.5-fold enrichment over non-fractionated poly(A)$^+$ RNA, not shown) was used to construct a directional cDNA library with the SuperScriptII cDNA synthesis system (GibcoBRL). cDNA pools (of . 2.5 H 10^4 clones each) were screened. A positive pool that increased transport activity to a degree similar to kidney cortex poly(A)$^+$ RNA was sequentially subdivided and analyzed until a single clone (SVCT1) was identified.

SVCT2 cDNA was isolated by PCR-based homology screening of a rat brain cDNA library. First-strand cDNA was prepared by reverse-transcription from rat brain poly(A)$^+$ RNA. Sense and antisense degenerate oligo-nucleotide primers were designed based upon the SVCT1 cDNA sequence (sense, ATH GAR TCN ATH GGN GAY TA, coding for amino acid residues 339-346 of SVCT1, and antisense, CC RAA RAA DAT NGA RAA NCC, residues 468-475) and used for PCR amplification of the brain cDNA. PCR products of the expected size (. 400 bp) were subcloned into the pCRII vector (Invitrogen) and sequenced. A single clone with 80% identity to SVCT1 was labelled using [^{32}P]-dCTP and used to screen a rat brain cDNA library constructed in the λgt10 vector (Stratagene). The library (. 4 H 10^5 clones) was screened under high stringency conditions by washing with 0.1 H SSC and 0.1% SDS at 65 EC for 1 h. A positive clone of a 6.5 kb cDNA was isolated and subcloned into pBluescript SK(!). The cDNA sequence was determined on both strands and analyzed using Genetics Computer Group tools. To improve expression in oocytes, an *Eco*RI site was introduced to the 5N-untranslated region (nucleotide 198) using PCR. Two 6.5-kb SVCT2 cDNA fragments obtained by *Eco*RI-*Eco*RI (1.5 kb) and *Eco*RI-*Sph*I (0.5 kb) digestions, were ligated and subcloned into a blunt-ended dephosphorylated *Not*I site in the vector pTLN2 (Ref. 2).

A rabbit cDNA (rbSVCT2) of 1.2-kb in length and with 96% identity to residues 96 to 505 of the rat SVCT2 was obtained by reverse transcription-PCR from rabbit brain mRNA using a set of rat SVCT2 oligonucleotide primers (*sense*, nucleotides 660-682; *antisense*, nucleotide 1870-1892). Sequence alignments were performed using the PILEUP program of Genetics Computer Group (Madison, WI).

Functional characterization of SVCT1 and SVCT2 in *Xenopus* oocytes. Oocytes were isolated from *Xenopus laevis* (under 2-aminoethylbenzoate anaesthesia), treated with collagenase A (Boehringer-Mannheim) and stored at 18 EC in modified Barths: medium[3]. Oocytes were injected with . 25 ng of SVCT1 or SVCT2 cRNA synthesized *in vitro*, or water only (control), and incubated 2-7 days before performing radiotracer or voltage-clamp experiments. Standard Na^+ uptake medium comprised 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.5 with Tris base). For Na^+-free or low-Na^+ media, NaCl was

replaced by equimolar choline chloride; likewise, Cl^- was replaced by gluconate for Cl^--free medium. For pH-sensitivity experiments, Na^+ media were buffered at pH 5.5-8.0 using 0-5 mM MES, 0-5 mM HEPES and 0-5 mM Tris base. L-Ascorbic acid-containing solutions were freshly-prepared (without dithiothreitol) and used immediately.

Radiotracer uptake was determined by incubating 6-10 oocytes for 30 or 60 min in standard Na^+ or Na^+-free media (at 22 °C) with 10-600 µM L-[1-^{14}C]ascorbic acid (NEN Life Science Products; final specific activity 0.3 GBq.mmol^{-1}). Oocytes were solubilized with 10% SDS and [^{14}C]-content was measured by liquid scintillation counting. A two-microelectrode voltage-clamp (Clampator CA-1B, Dagan Instruments) was used to measure currents (acquired using the Digidata 1200 interface and pCLAMP software, Axon Instruments) in control oocytes and oocytes injected with rat SVCT1 or rat SVCT2 cRNA. Microelectrodes (resistance 0.5-5 MΩ) were filled with 3 M KCl. Oocytes were superfused at 22 °C in standard Na^+ medium and clamped at a holding potential (V_h) of −50 mV. For continuous current monitoring at V_h = −50 mV, the current was low-pass filtered at 20 Hz (sampling at 1 Hz). Additionally, step-changes in membrane potential (V_m) were applied (from +50 mV to −150 mV in 20-mV increments), each for a duration of 100 ms, before and after the addition of substrate; current was low-pass filtered at 500 Hz and digitized at 5 kHz. Test solutions were always washed out with substrate-free medium (100 mM choline chloride) at pH 7.5 for several minutes. Steady-state data (obtained by averaging the points over the final 16.7 ms at each V_m step when step-changes were applied) were fitted to equation (1), for which I is the evoked current (*i.e.* the difference in steady-state current measured in the presence and absence of substrate), I_{max} the derived current maximum, S the concentration of substrate S (Na^+ or L-ascorbic acid), $K_{0.5}^S$ the substrate concentration at which current was half-maximal, and n_H the Hill coefficient for S. (For radiotracer experiments, I was replaced with velocity v and I_{max} replaced with the derived maximal velocity, V_{max}.)

$$I = \frac{I_{max} \cdot S^{n_H}}{\left(K_{0.5}^{S}\right)^{n_H} + S^{n_H}} \qquad (1)$$

Northern-blot analysis. Total RNA was isolated from rat tissues and cell culture of murine MC3T3-E1 osteoblasts[4] followed by oligo(dT) selection. Poly(A)$^+$ RNA (3 µg/well) was separated on a formaldehyde-agarose (7%/1%) gel and blotted onto a nitrocellulose filter. A 0.35-kb *Bgl*II digestion product from SVCT1 cDNA (nucleotides 425-773) and a 3.1-kb *Hin*dIII digestion product of SVCT2 cDNA (nucleotides 1-3101) were labelled with [^{32}P]-dCTP. The filters were hybridized at 42 °C in 50% formamide, and washed in 5 × SSC / 0.1% SDS at 50 °C for 2 × 30 min, then in 0.1 × SSC / 0.1% SDS at 65 °C for 3 × 20 min.

***In situ* hybridization.** Digoxigenin-labelled antisense and sense run-off transcripts were synthesized using the Genius Kit (Boehringer-Mannheim). Rat SVCT1 cRNA probes were transcribed from a PCR fragment that contained about 1.2 kb of SCVT1 cDNA (nucleotides 161-1411) flanked by promoter sites for SP6 and T7 polymerase. Rat SVCT2 cRNA probes were transcribed from a plasmid vector pBluescript SK(−) which contained 3.1 kb of the SVCT2 sequence (nucleotide 1 to 3101). Transcripts were alkali-hydrolyzed to an average length of 200-400 nucleotides. *In situ* hybridization was performed on 10-µm cryosections of fresh-frozen tissue as described[5]. Sections were immersed in slide mailers in hybridization solution of composition 50% formamide, 5 × SSC, 2% blocking reagent (Boehringer-Mannheim), 0.02% SDS and 0.1% *N*-laurylsarcosine, and hybridized at 70 °C for 16 h with probe concentrations of ~ 200 ng ml^{-1}. Sections were then washed 3 times in 2 × SSC and for 2 × 30 min in 0.2 × SSC at 70 °C. The hybridized labelled probes were visualized using anti-digoxigenin Fab fragments

(Boehringer-Mannheim) and BCIP/NBT substrate. Sections were developed in substrate solution for 16 h, rinsed in 100 mM Tris, 100 mM NaCl, 1 mM EDTA (pH 9.5), and coverslipped with Vectashield (Vector). For *in situ* hybridization of rbSVCT2 in rabbit, sense and antisense rbSVCT2 cRNA probes were prepared by RT-PCR based on the same oligonucleotide sequence from rat SVCT2 additionally flanked by the SP6 and T7 RNA polymerase promoter sequences. *In situ* hybridization was performed on 10-:m cryosections from the eye of a New Zealand albino rabbit (as described above for rat).

Hybrid depletion. Antisense oligonucleotides were generated against nucleotides 18-42 of rat SVCT1, and against nucleotides 383-405 of rat SVCT2. These were annealed (at 0.25 :g.:l^{B1}, in 50 mM NaCl at 42 EC) for 15 min with 0.5 :g.:l^{B1} poly(A)$^+$ RNA isolated from rat kidney cortex, small intestine or adrenal glands. Samples were then cooled on ice and injected into oocytes (. 25 ng/oocyte), before performing radiotracer assays as described above. The efficacy of the annealing process, and the suitability of the antisense oligonucleotides, was established using SVCT1 or SVCT2 cRNA: we found that annealing with the appropriate antisense oligonucleotide abolished L-ascorbic acid transport activity, with no appreciable cross-inhibition between SVCT isoforms (data not shown).

References to Supplementary Information

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