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Polarized localization of vitamin C transporters, SVCT1 and SVCT2, in epithelial cells

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Abstract

Messenger RNA of homologous sodium-vitamin C cotransporters, SVCT1 and SVCT2, were found in the intestine. Studies using cultured intestinal cells suggested an apical presence of SVCT1 but the function of SVCT2 was unknown. Here, we showed that enterocytes from heterozygous SVCT2-knockout mice had lower sodium-dependent vitamin C accumulation compared to those from the wildtype. Thus, SVCT2 appears to be functional in enterocytes. We then tested whether SVCT2 could have a redundant function as SVCT1 by constructing and expressing EGFP-tagged SVCTs in intestinal Caco-2 and kidney MDCK cells. In confluent epithelial cells, SVCT1 protein expressed predominantly on the apical membrane. SVCT2, in contrast, accumulated at the basolateral surface. Functionally, SVCT1 expression led to more transport activity from the apical membrane, while SVCT2 expression only increased the uptake under the condition when basolateral membrane was exposed. This differential epithelial membrane distribution and function suggests non-redundant functions of these two isoforms.

Keywords: Vitamin C; SVCT1; SVCT2; EGFP; Intestine; Kidney

Vitamin C (ascorbic acid) is an essential enzyme cofactor and also acts as a water-soluble antioxidant. Two sodium-dependent transporters for vitamin C, SVCT1 and SVCT2, have been cloned [1] and they share 66% amino acid homology. Although most species including mouse can synthesize vitamin C in the liver from glucose, homozygous SVCT2 knock-out mice died immediately after birth [2] indicating that this transporter could be universally obligatory for maintaining vitamin C homeostasis.

Both SVCT1 and SVCT2 were found in the intestine based on the mRNA analysis [1,3–5]. Previous studies of intestinal vitamin C transport using human intestinal epithelial cell line, Caco-2, focused exclusively on SVCT1. Vitamin C transport measurement and imaging analysis supported the presence of SVCT1 in the apical membrane of Caco-2 cells [6,7]. The functional significance of SVCT2 in enterocytes was unknown and the membrane distribution of SVCT2 in epithelial cells was never reported.

In this study, we used mouse intestine and cultured Caco-2 cells to examine the functional presence of SVCT2 in intestinal epithelial cells. In addition, we used live cell confocal fluorescence imaging to monitor the contrasting distribution of these two transporters in the intestinal and renal epithelial cells in both pre-confluent and post-confluent stages. Together with functional analysis, our data suggest non-redundant functions of these two transporters.

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Materials and methods

Cells and chemicals. Human intestinal Caco-2 cells were purchased from ATCC. Parental MDCK cell line was a gift from Dr. Mary Taub, University at Buffalo. Parental CHO cell line was a gift from Dr. John Subject, Roswell Park Cancer Institute. All cells were propagated following a published method using DMEM with 10% FBS in 5% CO_2 chamber [8]. All experiments using stably transfected cells were performed within the first 10 passages of each stable line. Cultured cell experiments were performed in triplicate wells and were repeated at least twice. Intestinal tissue and primary enterocytes were derived from 10- to 12-weeks-old *Slc23a2*+/+ and *Slc23a2*+/− mice [2]. Enterocyte collection was performed following an established low calcium method with sequential citrate and EDTA treatments [9]. The integrity of the isolated enterocytes was confirmed by a greater than 90% dye exclusion. L-[Carboxyl-^{14}C]ascorbic acid (13 mCi/mmol) for the transport activity measurement was from Amersham Biosciences (Piscataway, NJ). All other chemicals used were of reagent grade.

Transport experiment. Uptake of [^{14}C]ascorbic acid by cell monolayers grown on six-well culture plates or on Transwell (#3450, Corning Science Products) was carried out at 37 °C in the presence of Hanks' balanced salt solution following published methods [10–12]. Freshly isolated primary enterocytes and cell suspensions obtained by trypsinizing cell monolayers were used for the measurement of [^{14}C]ascorbic acid uptake at 37 °C in the presence of Hanks' balanced salt solution following an established rapid filtration method [9]. The radioactivity retained by the Millipore DAWP filter was then counted by the liquid scintillation technique. Our previous experiments have shown that uptake results from measuring ^{14}C radioactivity were consistent with results from direct ascorbic acid determination by HPLC [13].

RT-PCR. The extraction of RNA from intestinal epithelia, RT reaction, and semi-quantitative PCRs was performed following published methods [14,15]. Published primer sets were used for the PCR of mouse enterocytes [15].

Plasmid construction and transfection. PGEM-Teasy plasmids containing complete coding sequences of human SVCT1 and SVCT2 between two *Eco*RI sites were generous gifts of Dr. Mark Levine of NIH [3]. The coding sequences of the two transporters were modified by PCR at the C-termini to change the stop codon to GCA (SVCT1) or TCC (SVCT2) and to create *Bam*HI sites. The sequences were then inserted by restriction enzyme digest and three-way ligation in-frame into the multicloning site of pEGFP-N2 (B-D Biosciences) between the *Eco*RI and *Bam*HI sites. The resulting pSVCT1-EGFP and pSVCT2-EGFP constructs have identical linking sequence before the enhanced green fluorescence protein (EGFP) sequence in C-termini. DNA sequencing (Roswell Park Cancer Institute DNA sequencing facility) was performed to confirm the integrity of the coding sequences of the SVCT1-EGFP and SVCT2-EGFP chimeric constructs. Transfections of plasmids to various cell lines and the establishment of stable cell lines were carried out by a published calcium phosphate precipitation method [16,17]. The established stably transfected cell lines were termed Caco-2-SVCT1, Caco-2-SVCT2, MDCK-SVCT1, MDCK-SVCT2, CHO-SVCT1, and CHO-SVCT2 to represent their original cell lineages and the genes over-expressed.

Wide-field and confocal fluorescence microscope imaging. Cells grown on the Lab-Tek II chamber coverglass system (Nalge Nunc International) were used for the imaging experiments. They were visualized in a 37 °C microscope incubation chamber in the presence of Hanks' balanced salt solution supplemented with 0.5% FBS. A Nikon Diaphot fluorescence microscope was used either with a mercury arc light source in wide-field mode or as part of the Bio-Rad MRC-1024 laser confocal microscope system. For wide-field fluorescence observation, a Zeiss 63× NA1.4 oil-immersion objective lens was used along with a 450–490 nm excitation filter and 520 nm longpass emission filter. Images were captured with a Xylix PMI-1400 cooled CCD camera (QImaging, Burnaby, B.C. Canada). Confocal images were acquired with a Nikon 60× NA1.4 objective and used the 488 nm laser line for excitation with an emission bandpass filter of 522 ± 16 nm. All scanning was carried out from the basolateral side (bottom of the cells) to the apical side (top of the cells) covering only sections where the fluorescence can be detected. The confocal images were processed with Velocity 2.6 (Improvision, Lexington, MA) and NIH ImageJ softwares.

Results

Functional presence of SVCT2 in mouse enterocytes

The presence of mRNA of SVCT1 and SVCT2 in the intestinal epithelium was demonstrated previously [1,5] but the functional presence of SVCT2 in the intestinal epithelium was not known. To examine the presence of functional SVCT2 in the intestinal epithelium, primary enterocytes from heterozygous SVCT2 knockout mice were used as a model. Homozygous knockout of SVCT2 leads to death right after birth [2]. As shown in Fig. 1A, enterocytes isolated from the ileum of SVCT2+/− mice had significantly reduced sodium-dependent vitamin C accumulation compared to enterocytes from SVCT2+/+ mice. This finding supports the functional presence of SVCT2 in enterocytes. Results from the RT-PCR analysis of intestinal epithelium (Figs. 1B and C) further confirmed the contribution of SVCT2. The mRNA level of SVCT2 in the intestinal epithelium was reduced in SVCT2+/− mice (Fig. 1B) but the level of SVCT1 mRNA remained unchanged (Fig. 1C).

We also used human intestinal cell line, Caco-2, which can undergo spontaneous differentiation [18], as an enterocyte model. Similar to the intestinal epithelium observation above, mRNA of SVCT1 and SVCT2 were both detected in differentiated Caco-2 cells (Fig. 2A). We found that Caco-2 cells grown on the permeable support can take in vitamin C from the apical membrane as well as the basolateral membrane (Figs. 2B and C). Net sodium-dependent transport of vitamin C across the basolateral membrane reached its saturation at around 100 μM ascorbic acid (Fig. 2B) but the transport across the apical membrane had a lower affinity (Fig. 2C). The observation suggests the presence of different mechanisms for the vitamin C transport across different membranes of polarized intestinal epithelial cells. It has been shown that SVCT1 has a lower affinity for vitamin C compared to SVCT2 [3,19,20].

Construction and characterization of hSVCT1-EGFP and hSVCT2-EGFP plasmids

To further determine whether SVCT1 and SVCT2 could have a redundant function in enterocytes, we decided to examine the distribution of SVCT1 and SVCT2 in polarized epithelial cells. A difference in

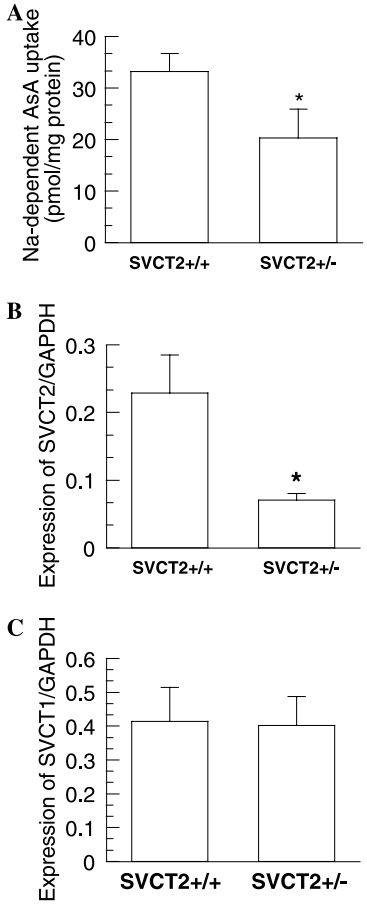

Fig. 1. Vitamin C transport activity and gene expression by enterocytes from SVCT2+/+ and SVCT2+/− mice. (A) Net sodium-dependent (uptake in the presence of sodium minus uptake in the absence of sodium) vitamin C transport activity of freshly isolated enterocytes. Means ± SD and $N = 3$ enterocyte preparations for each genome type. (B,C) Comparison of mouse intestine mRNA levels of SVCT2 (B) and SVCT1 (C) as determined by semi-quantitative RT-PCR. Levels of GAPDH were used for the normalization. Means ± SE and $N = 12$ for SVCT2+/+ genome type; $N = 15$ for SVCT2+/− genome type. *Significantly different from the SVCT2+/+ at $p < 0.05$ by Student's t test.

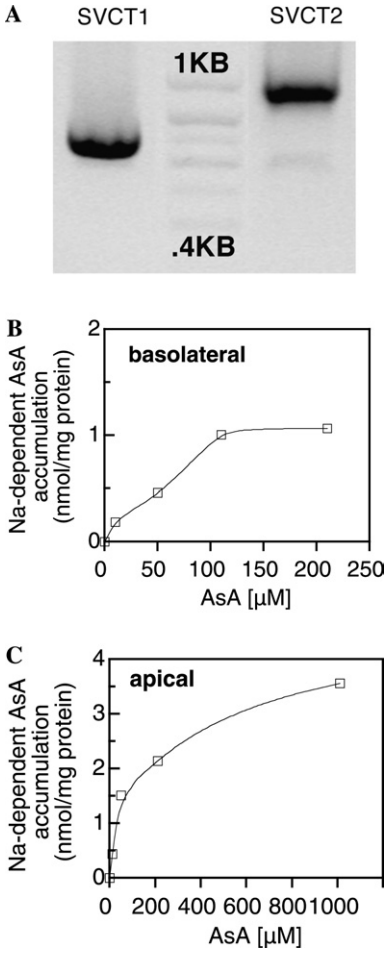

Fig. 2. Concentration-dependent uptake of vitamin C into post-confluent human intestinal cells, Caco-2, grown on the permeable support of Transwell. (A) Expression of SVCT1 and SVCT2 in Caco-2 cells demonstrated by RT-PCR. Sodium-dependent vitamin C transport across the (B) basolateral membrane, (C) apical membrane, into cells during the 30-min incubation.

membrane distribution will suggest non-redundant functions. We constructed SVCT1 and SVCT2 expression plasmids with EGFP attached to the C-termini, pSVCT1-EGFP and pSVCT2-EGFP. These constructs were characterized initially in non-polarizing CHO cells. As shown in Figs. 3A and B, these chimeric proteins can localize to the cell membrane although some intracellular localization of SVCT1 was also observed as previously reported [7,21]. Similar to previously reported [22], cells transfected with the parental EGFP plasmid without SVCT1 or SVCT2 sequence showed predominantly cytosolic and nuclear localization (results not shown). There was no endogenous fluorescence under our experimental condition.

These membrane-localized chimeric proteins are also functional. In stably transfected CHO cells, SVCT1-EGFP and SVCT2-EGFP increased sodium-dependent vitamin C accumulation but had little effect on the sodium-independent vitamin C accumulation (Fig. 3C). Kinetic analysis of these stably transfected CHO cells revealed an apparent ascorbic acid K_m of 103 μM for SVCT1 and 14.6 μM for SVCT2 (Fig. 3D), which are consistent with previous reports on untagged and V5-tagged human SVCT1 and SVCT2 [3,21]. We have shown that estrogens can dose-dependently inhibit sodium-dependent vitamin C accumulation in Caco-2 and other cells [11]. Here, synthetic estrogen, diethylstilbestrol (DES), inhibited the vitamin C accumulation mediated by SVCT1-EGFP and SVCT2-EGFP in stably transfected CHO cells in a dose-dependent fashion (Fig. 3E).

Polarized distribution of SVCT1 and SVCT2 in epithelial cells

Despite epithelial origin, CHO cells are not polarized and do not undergo differentiation [23]. To determine

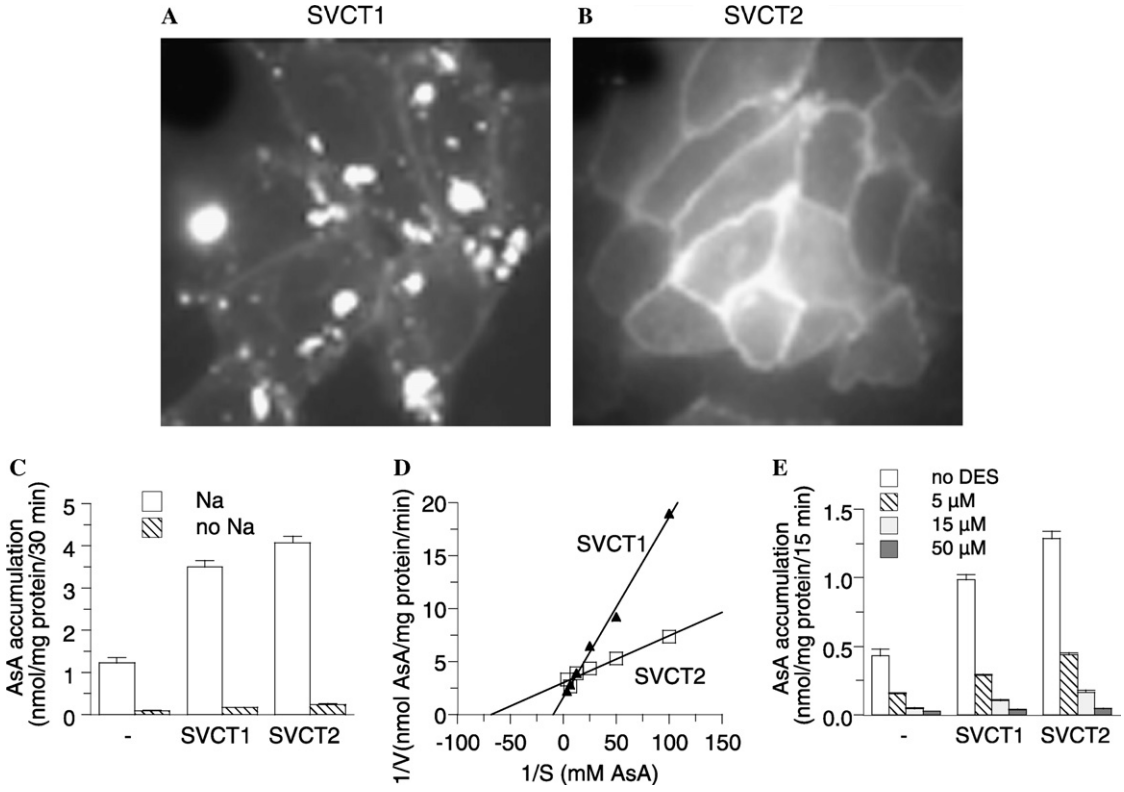

Fig. 3. Localization and functional characterization of SVCT1-EGFP and SVCT2-EGFP constructs in CHO cells. (A,B) Localization of EGFP-tagged SVCT1 (A) and SVCT2 (B) in stably transfected non-differentiating CHO cells under wide-field fluorescence microscope. (C) Vitamin C uptake in the presence or absence of sodium by CHO cells stably transfected with SVCT1-EGFP or SVCT2-EGFP. (D) Determination of apparent K_m and V_{max} for SVCT1-EGFP and SVCT2-EGFP in stably transfected CHO cells in a 15-min uptake. The vitamin C transport of the parental CHO cells were subtracted from that of the transfected cells before the calculation. For SVCT1, $K_m = 103$ μM, $V_{max} = 0.604$ nmol/mg protein/min; for SVCT2, $K_m = 14.6$ μM, $V_{max} = 0.332$ nmol/mg protein/min. (E) Diethylstilbestrol inhibition of SVCT1-EGFP and SVCT2-EGFP-mediated vitamin C accumulation in CHO cells. Means ± SD and $N = 3$ for all data points.

whether SVCT1 and SVCT2 have differential localization in epithelial cells, we used SVCT1- and SVCT2-stably transfected Caco-2 cells as the model. Prior to reaching confluence, SVCT1 was localized in the intracellular compartments as well as on the apical membrane (Figs. 4A and E). In comparison, post-confluent Caco-2 cells that had extensive dome formation as observed under the phase microscope expressed SVCT1 mostly on the apical membrane (Figs. 4B and F). SVCT2 distribution also changed with the cell differentiation. Initially, SVCT2 was found on the basal and lateral surface of the cells (Figs. 4C and G). It localized mostly in the basal membrane focal adhesion-like area in post-confluent Caco-2 cells (Figs. 4D and H).

Polarized localization of SVCT1 and SVCT2 was also observed in stably transfected kidney MDCK cells (Fig. 5). The distribution patterns were similar between Caco-2 cells and MDCK cells (Fig. 4 vs. Fig. 5). Pre-confluent MDCK cells had more SVCT1 in the intracellular compartment (Figs. 5A and E), while post-confluent MDCK cells that showed extensive dome formation as observed under the phase microscope had SVCT1 mostly on the apical membrane (Figs. 5B and F). SVCT2 distribution, in addition to distinctively different from SVCT1, also changed from basal and lateral distribution in the pre-confluent cells to intensively basal localization in post-confluent cells (Figs. 5C, D, G, and H).

Functional non-redundancy of expressed SVCT1 and SVCT2

Functional transport assay was used to further validate the polarized distribution of SVCT1 and SVCT2 in the epithelial cells. If SVCT1 and SVCT2 have non-redundant functions, they will only mediate sodium-dependent vitamin C transport from the membranes that they reside. Post-confluent Caco-2-SVCT1 and MDCK-SVCT1 cells had increased sodium-dependent vitamin C transport activity when the transport assay was performed with intact monolayers (only apical membrane exposed) (Figs. 6A and C). In contrast, post-confluent Caco-2-SVCT2 and MDCK-SVCT2 cells did not have increased sodium-dependent vitamin C transport activity when assayed as intact monolayers (Figs. 6A and C). After cells were dissociated from the solid support (thus exposing the basolateral membrane),

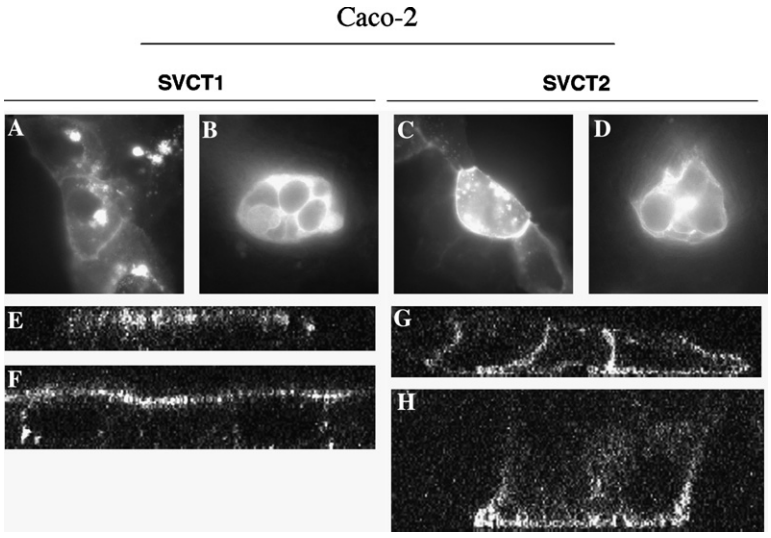

Fig. 4. Polarized and differentiation-dependent localization of SVCT1 and SVCT2 in Caco-2 cells as determined by cells stably transfected with EGFP-tagged transporters. (A–D) Imaging of stably transfected Caco-2 cells under wide-field fluorescence microscope; (E–H) X, Z-section of stably transfected Caco-2 cells under confocal microscope. The top of the figure is the apical side, while the bottom of the figure is the basolateral side. (A,E,C,G) Days 3–5 after seeding when monolayers were not completely confluent; (B,F,D,H) post-confluent at day 16 after seeding.

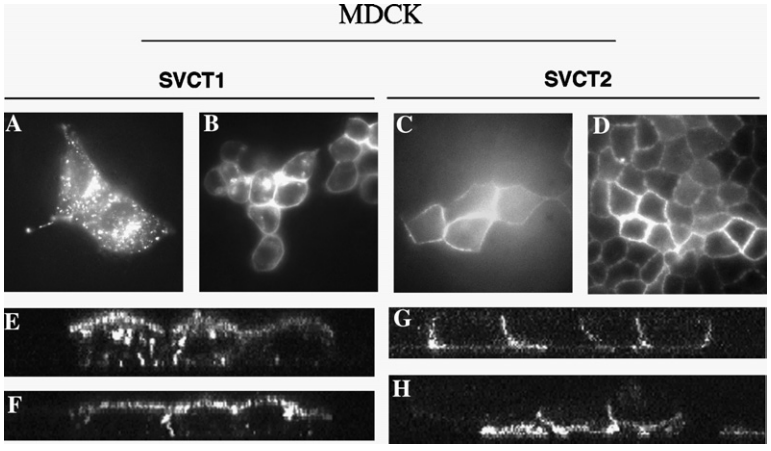

Fig. 5. Polarized and differentiation-dependent localization of SVCT1 and SVCT2 in MDCK cells as determined by cells stably transfected with EGFP-tagged transporters. (A–D) Imaging of stably transfected MDCK cells under wide-field fluorescence microscope; (E–H) X, Z-section of stably transfected MDCK cells under confocal microscope. The top of the figure is the apical side, while the bottom of the figure is the basolateral side. (A,E,C,G) Day 3 after seeding when monolayers were not completely confluent; (B,F,D,H) post-confluent at day 13 after seeding.

enhanced sodium-dependent transport activity can be observed for Caco-2-SVCT2 and MDCK-SVCT2 cells (Figs. 6B and D). Furthermore, when MDCK cells were grown on the permeable support, stable expression of SVCT1-EGFP only led to an increase in the vitamin C accumulation from the apical chamber, while stable expression of SVCT2-EGFP only led to an increase in the vitamin C accumulation from the basolateral chamber (Figs. 6E and F).

Discussion

SVCT1 was proposed to handle the bulk transport of apical vitamin C in the intestine when it was first cloned [1]. The preferential apical localization, as shown here by both transport assay and visualization (Figs. 4–6), is consistent with the model. The establishment of SVCT1 knockout mice will help to conclude the essentiality of SVCT1 for the intestinal absorption of vitamin C from the diet. The function of SVCT2 in the intestine was not examined previously. In this study, we used enterocytes from heterozygous SVCT2-knockout mice to demonstrate the functional presence of SVCT2 in the intestine. Enterocytes from SVCT2+/− mice showed a significantly lower sodium-dependent vitamin C transport activity (Fig. 1A). This change in the transport activity was specifically correlated with changes in the SVCT2 mRNA level (Fig. 1B), since the level of SVCT1 mRNA remained unchanged in the SVCT2+/− mice (Fig. 1C).

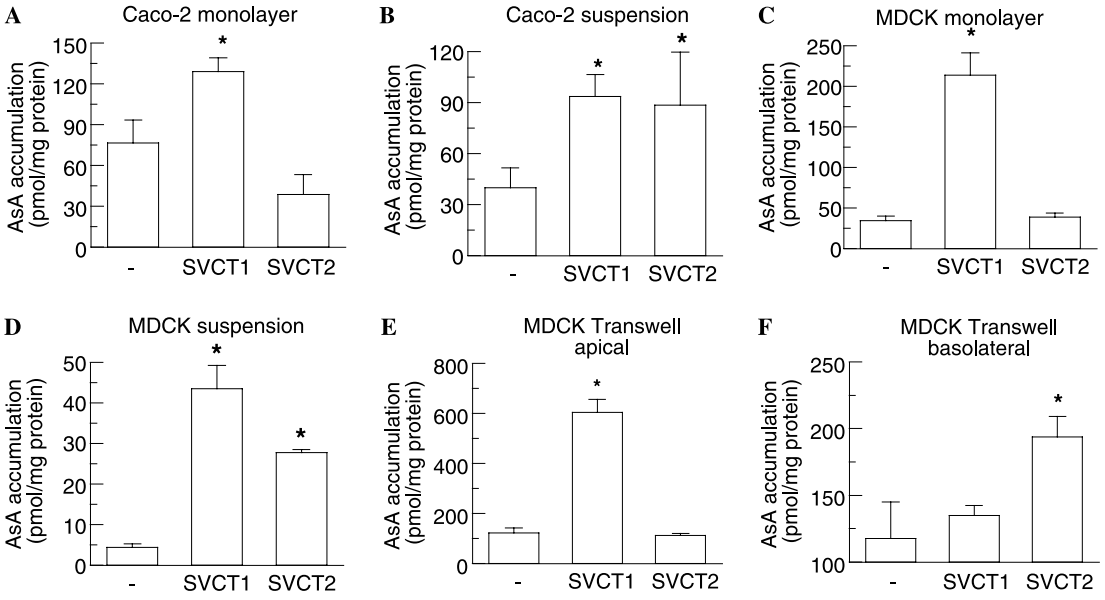

Fig. 6. Transport of vitamin C by parental and transfected cells as post-confluent monolayers or cell suspensions derived from the post-confluent monolayers. (A,B) Caco-2, Caco-2-SVCT1 and Caco-2-SVCT2 cells; (C–F) MDCK, MDCK-SVCT1, and MDCK-SVCT2 cells. (A,C) Net sodium-dependent uptake of vitamin C (uptake in the presence of sodium minus uptake in the absence of sodium) by cells grown on six-well plates and uptake of [^{14}C]ascorbic acid measured as monolayer. (B,D) Net sodium-dependent uptake of [^{14}C]ascorbic acid by cell suspensions trypsinized from monolayers grown on the solid support. (E,F) Net sodium-dependent accumulation of [^{14}C]ascorbic acid by MDCK cell lines grown on Transwells. [^{14}C]ascorbic acid was only added to the apical chamber (E) or basolateral chamber (F). Means ± SD and $N = 3$ for all data points. One-way analysis of variance (ANOVA) and post hoc multiple comparison (Bonferroni/Dunnett's multiple range test comparing to the parental line) were performed. *Significantly different from the parental line at $p < 0.05$.

The next question is whether SVCT2 could have a redundant function as SVCT1 in enterocytes, i.e., both transporters are involved in the absorption of vitamin C from the apical membrane. Differences in the substrate affinity of apical and basolateral membranes (Fig. 2) suggested that differentiated Caco-2 cells use different mechanisms for vitamin C transport across these two membranes. Experiments using EGFP-tagged SVCT1 and SVCT2 provided more convincing evidences. SVCT1 and SVCT2 have different membrane localizations in epithelial cells although the rest of their constructs were identical (Figs. 4 and 5). They also mediate vitamin C transport across different membranes (Fig. 6). Their functions thus are unlikely to be redundant. The preferential basolateral localization of SVCT2 in epithelial cells as shown here suggested that SVCT2 may not contribute to the intestinal absorption of dietary vitamin C. For species that synthesize vitamin C endogenously and do not routinely have dietary vitamin C intake, the ability to transport vitamin C from blood across the basolateral membrane into enterocytes is physiologically important. Based on this study, SVCT2 is a candidate gene for the process.

In all the three cell lines we examined, SVCT1, in addition to its membrane localization, is also found in the intracellular compartment based on both wide-field and confocal microscopic observation (Figs. 3–5). Similar intracellular localization was reported for SVCT1-YFP [7]. This intracellular SVCT1 localization has been previously demonstrated in fibroblast-like COS-1 cells where the SVCT1 protein was tagged with the V5 epitope and visualized by immunocytochemistry [21]. Thus, the protein localization was unlikely an artifact due to EGFP. We followed the same passage of cells from the pre-confluent to the well-differentiated stage (Figs. 4 and 5). In both Caco-2 cells and MDCK cells, the intracellular distribution was reduced significantly in well-differentiated cells. Our results suggest that the intracellular compartment is an intermediate site in protein processing for SVCT1. This is possible considering the complicated events involved in the membrane protein processing [24]. Membrane to intracellular shuttling was proposed as the mechanism behind the phosphorylation-induced reduction in SVCT1-mediated vitamin C transport [21]. Although DES can dose-dependently reduce vitamin C accumulation (Fig. 4E), we did not observe any changes in SVCT1 localization when cells were exposed to DES. DES also did not change the membrane localization of SVCT2 (results not shown).

SVCT1 and SVCT2 share 66% amino acid homology, yet they are sorted to distinct membrane compartments in epithelial cells. This pair of proteins could serve as a model for the understanding of protein sorting. PDZ binding domain was implied in the sorting of apical membrane proteins [25–27]. Although SVCT1 has a putative PDZ binding domain CTKV in the C terminal, this domain is unlikely to be important for the preferential sorting. Previous study [7] found that partial

C-terminal deletion of SVCT1, including the removal of CTKV, had no effect on the membrane localization. Further deletion/mutation is needed to determine the domains necessary and sufficient for the differential membrane sorting.

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