Up-regulation and Polarized Expression of the Sodium-Ascorbic Acid Transporter SVCT1 in Post-confluent Differentiated CaCo-2 Cells*

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Human cells acquire vitamin C using two different transporter systems, the sodium-ascorbic acid co-transporters with specificity for ascorbic acid, and the facilitative glucose transporters with specificity for dehydroascorbic acid. There is no information on the mechanism of vitamin C transport across the intestinal barrier, a step that determines the bioavailability of vitamin C in humans. We used the colon carcinoma cell line CaCo-2 as an *in vitro* model for vitamin C transport in enterocyte-like cells. The results of transport kinetics, sodium dependence, inhibition studies, and reverse transcriptase-PCR analysis indicated that CaCo-2 cells express the sodium-ascorbate co-transporters SVCT1 and SVCT2, the dehydroascorbic acid transporters GLUT1 and GLUT3, and a third dehydroascorbic acid transporter with properties expected for GLUT2. Analysis by real time quantitative PCR revealed that the post-confluent differentiation of CaCo-2 cells was accompanied by a marked increase (4-fold) in the steady-state level of SVCT1 mRNA, without changes in SVCT2 mRNA levels. Functional studies revealed that the differentiated cells expressed only one functional ascorbic acid transporter having properties expected for SVCT1, and transported ascorbic acid with a V_{max} that was increased at least 2-fold compared with pre-confluent cells. Moreover, post-confluent Caco-2 cells growing as monolayers in permeable filter inserts showed selective sorting of SVCT1 to the apical membrane compartment, without functional evidence for the expression of SVCT2. The identification of SVCT1 as the transporter that allows vectorial uptake of ascorbic acid in differentiated CaCo-2 cells has a direct impact on our understanding of the mechanism for vitamin C transport across the intestinal barrier.

Vitamin C is an essential micronutrient required for the maintenance of a normal human physiology. Vitamin C is required for the synthesis of collagen and carnitine, as a cofactor in enzymes to maintain metal ions in their reduced form, and to protect tissues from oxidative damage by scavenging free radicals (1–6). Also, reduced vitamin C (ascorbic acid) can recycle glutathione and vitamin E, two important biologic antioxidants. Recent evidence (7) indicates that an increased intake of vitamin C is associated with a reduced risk of chronic diseases such as cancer or cardiovascular disease.

Vitamin C exists in two chemically distinct forms in human plasma, the reduced ascorbate ion form (ascorbic acid (AA)[1] and the oxidized non-ionic form (dehydroascorbic acid (DHA)). Human cells acquire both chemical forms of vitamin C by transporting them across the cell membrane with the participation of two different transporter systems that show absolute specificity for one or the other vitamin form (2, 8, 9). One transporter system behaves as a low affinity, high capacity sodium-independent system and includes several members of the facilitative glucose transporter family (GLUTs) (10–13). These transporters show a high specificity for oxidized vitamin C and transport dehydroascorbic acid down a substrate concentration gradient (14–17). Twelve glucose transporter isoforms have been molecularly characterized, and there is evidence that the isoforms GLUT1, GLUT3, and GLUT4 are efficient dehydroascorbic acid transporters. A second transport system for vitamin C is a high affinity, low capacity sodium-dependent system (SVCTs) that corresponds to a recently described family of mammalian sodium-ascorbic acid co-transporters composed of two members, SVCT1 and SVCT2. These transporters display high affinity for reduced vitamin C (9, 18–21).

Because humans are not capable of synthesizing vitamin C, it must be obtained from the diet and then acquired by the different body cells. Although we know the molecular identities of the vitamin C transporters expressed in human cells (9, 13, 16, 17, 20–22), there is no information regarding the identity and functional properties of the cellular mechanisms that control vitamin C transfer across the intestinal barrier and the bioavailability of vitamin C in humans. Supplementation studies in normal volunteers have determined that the step limiting vitamin C bioavailability in humans lies at the transcellular transport within the intestine (23). However, we know very little regarding the most basic aspects of this process, including how vitamin C enters the apical membrane of the intestinal

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[1] The abbreviations used are: AA, ascorbic acid; DHA, dehydroascorbic acid; RT, reverse transcriptase; GLUT, glucose transporter; SGLT, sodium-glucose co-transporter.

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epithelium, whether it is processed intracellularly, or how it exits the basolateral membrane (1, 2, 24–29).

We used the human colon carcinoma cell line CaCo-2 as an *in vitro* model for vitamin C transport in enterocyte-like cells. CaCo-2 cells have been extensively used to study the transcellular movement of nutrients, drugs, or metal ions *in vitro* because they differentiate in culture, both structurally and functionally, resembling mature enterocytes (30–34). These cells have been successfully used to study glucose transport regulation and to identify the molecular components by which glucose crosses the intestinal barrier. These studies revealed that CaCo-2 cells express several glucose transporters such as the Na^+-glucose co-transporter SGLT, facilitative glucose transporters GLUT1, GLUT2, and GLUT3, and the fructose transporter GLUT5. It was also shown that the polarized distribution of the different transporters at the apical or basolateral membrane compartments is central to the capacity of these cells to transport glucose in a vectorial manner, *i.e.* apical to basolateral direction (24, 26, 35, 36).

We present data indicating that cultured CaCo-2 cells transport both reduced and oxidized vitamin C. Transport analysis, together with competition and inhibition studies and RT-PCR analysis, revealed that vitamin C uptake by CaCo-2 cells is mediated by two transporter families. We conclude that CaCo-2 cells express the sodium-ascorbate co-transporters SVCT1 and SVCT2, the dehydroascorbic acid transporters GLUT1 and GLUT3, and a third dehydroascorbic acid transporter with properties expected for GLUT2. Moreover, our data indicate that SVCT transporter expression is regulated during *in vitro* differentiation of CaCo-2 cells. Post-confluent differentiated CaCo-2 cells showed increased expression of SVCT1 at RNA and protein levels, and this increased expression was accompanied by selective sorting of SVCT1 to the apical membrane compartment. Our data have a direct impact on our understanding of the mechanism for vitamin C transport at the intestinal level, as well as on the vectorial transport of the vitamin across the intestinal barrier.

EXPERIMENTAL PROCEDURES

CaCo-2 cells were used at passages 20–60 from stock cells grown in T75 plastic flasks. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, penicillin/streptomycin, and fungizone. Cells were dispersed by treatment with 0.25% trypsin, 5 mM EDTA at 80% confluence (31, 34). Cell viability was always greater than 95% as assessed by trypan blue exclusion. Tissue culture media, supplements, and tissue culture reagents were obtained from Invitrogen.

Vitamin C uptake experiments were performed in 6-well plates containing 1×10^6 cells/well. For uptake assays, the cells were deprived of fetal bovine serum, growth factors, and other media supplements by replacing the culture media with incubation buffer (15 mM Hepes (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, and 0.8 mM MgCl$_2$) for 1 h before performing the assays. Uptake was done in incubation buffer containing labeled ascorbic acid or dehydroascorbic acid. Ascorbic acid transport assays were performed in 0.6 ml of incubation buffer containing 0.1–0.4 μCi of L-[^{14}C]ascorbic acid (specific activity 8.2 mCi/mmol, PerkinElmer Life Sciences) at a final concentration of 5–500 μM ascorbic acid (Sigma) in the presence of 0.1 mM dithiothreitol. For dehydroascorbic acid uptake, 1–10 units of ascorbic acid oxidase (50 units/mg protein, Sigma) was added to the ascorbic acid mixture and incubated for 5 min at 37 °C before adding it to the cells at a final concentration of 0.5–12 mM. Uptake was finished by adding 5 volumes of cold stopping solution (NaCl 160 mM, KCl 5 mM, MgSO$_4$ 0.8 mM, CaCl$_2$ 1.8 mM, HgCl$_2$ 0.2 mM) (37). The cells were washed twice with cold phosphate-buffered saline (pH 7.4) and lysed in 300 μl of 10 mM Tris-HCl (pH 8.0) containing 0.2% SDS, and the incorporated radioactivity was determined by liquid scintillation counting (38). Transport in the absence of sodium ions was accomplished by replacing the NaCl in the incubation media with 135 mM choline chloride (Sigma). When appropriate, competitors (deoxyglucose, fructose, sucrose, and α-methyl-D-glucopyranoside) and inhibitors (phloretin or genistein) were added to the uptake assays simultaneously with the transported sub-

strate, or the cells were preincubated in their presence prior to the uptake assay (cytochalasin B and cytochalasin E). Hexose uptake assays were similarly performed using 1 μCi of 2-[1,2-^{3}H]deoxy-D-glucose (specific activity 26.2 Ci/mmol, PerkinElmer Life Sciences) and 0.1–50 mM 2-deoxy-D-glucose (deoxyglucose) or 1 μCi of 3-O-[*methyl*-^{3}H]D-glucose (specific activity 10 Ci/mmol, PerkinElmer Life Sciences) and 0.1–50 mM 3-O-methyl-D-glucose (methylglucose). Time course experiments measuring the transport of methylglucose, a nonmetabolizable glucose analog, showed that the rate of transport for 1 mM substrate was linear for the first 60 s, with an equilibrium being reached in about 30 min. This allowed us to estimate an intracellular water-exchangeable volume of 2 μl/10^6 cells.

For RT-PCR, CaCo-2 total mRNA was isolated from cells growing in monolayers ($\sim 5 \times 10^6$ cells) with the Micro Poly(A) Pure^{TM} kit (Ambion) according to the manufacturer's instructions. Total human brain RNA was obtained from Clontech. For both human brain and CaCo-2 cells, cDNA synthesis was performed by RT-PCR amplification using the Advantage^{TM} RT kit (Clontech) following the manufacturer's instructions. Expression of hSVCT1 and hSVCT2 transporters in CaCo-2 cells and total human brain was verified by PCR. Oligonucleotide primer pairs for SVCT1 (forward primer, Fsvct1–733, 5′-ACTCTCCTCCG-CATCCAGAT-3′; reverse primer, Rsvct1–1017, 5′-CCAGGCGGGCA-CAGGCGTAG-3′) and SVCT2 (Fsvct2–1025, 5′-AGTATGGCTTCTAT-GCTCGC-3′; Rsvct2–1464, 5′-TTCCGGATCCTGTGCTGGA-3′; or Fsvct2–733, 5′-TTGACCATTACACCCACGGT-3′; and Rsvct2–1020, 5′-CATAGAAGCCATACTTTGTG-3′) were designed based on GenBank^{TM} sequence accession numbers AJ269477 and AJ269478, respectively. PCR amplification was done using 4 μl of cDNA template (diluted 1:100), 0.3 μM of each primer, 1 unit *Taq* polymerase (Invitrogen), 1× PCR buffer, 2.5 mM MgCl$_2$ (Invitrogen), 250 μM dNTP mix (Roche Molecular Biochemicals), and the following set of reactions: 1) denaturation step, 4 min at 94 °C; 2) 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min 10 s at 72 °C; and 3) 7 min at 72 °C after the last cycle. The PCR products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. The 304-, 459-, and 307-bp PCR products were extracted and purified from agarose gels with the QIAX Kit (Qiagen), cloned in pBluescript II KS (Stratagene), sequenced, and analyzed by BLAST using the NCBI server at www.ncbi.nlm.nih.gov/.

For quantitative RT-PCR, total RNA was isolated from CaCo-2 cells cultured in monolayers for 1–20 days (seeded at 3–4 × 10^5 cells/well in wells coated with collagen from rat tails) by using the SV Total RNA Isolation System^{TM} (Promega) according to the manufacturer's instructions. cDNA synthesis was done using the 1st strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus, Roche Molecular Biochemicals) and 1 μg of total RNA using the oligonucleotide primer pairs described above. Each PCR amplification product corresponding to 304 and 307 bp (for SVCT1 and SVCT2, respectively) was used as the standard for quantitative RT-PCR. Amplification of β-actin was used as the internal control. PCR amplification was done with the Light Cycler (Roche Molecular Biochemicals) using 2 μl of cDNA template, 0.5 μM each primer, 2–3 mM MgCl$_2$ (Invitrogen), and 2 μl of Fast-Start mix (Roche Molecular Biochemicals) in a final volume of 20 μl under the following reaction conditions: 1) 10 min at 95 °C; 2) 40 cycles of 5 s at 95 °C, 5–10 s at 65–55 °C (temperature gradient), and 15–20 s at 72 °C; 3) a final cycle of 5 min at 72 °C. PCR products were analyzed by 1% agarose gel electrophoresis and stained with SYBR Green (Sigma).

Data are presented as the average $\pm$ S.D. and correspond to a minimum of three assays performed independently in triplicate. Kinetic parameters were determined using the Michaelis-Menten equation and by using the linear transformation of Eadie-Hofstee. In experiments that revealed the presence of more than one kinetic component, K_m and V_{max} data were corrected by using successive iterations (39).

RESULTS

Time course analysis of vitamin C uptake revealed that CaCo-2 cells take up both reduced and oxidized vitamin C but showed clear quantitative differences in their capacity to incorporate both forms of the vitamin (Fig. 1*A*). The rate of dehydroascorbic acid incorporation (820 pmol/min per 10^6 cells) was 10-fold greater than the rate of ascorbic acid incorporation (80 pmol/min per 10^6 cells). Uptake of ascorbic acid was sodium-dependent, as shown by a greater than 90% decrease in the rate of uptake (to 1.5 pmol/min per 10^6 cells) when choline chloride replaced NaCl in the incubation buffer (Fig. 1*B*). In contrast, the rate of dehydroascorbic acid was similar in the presence

FIG. 1. **Uptake of vitamin C in CaCo-2 cells.** *A*, time course of AA (●) or DHA (○) uptake. *B*, ascorbic acid uptake in the presence (○) or in the absence (●) of NaCl (replaced with choline chloride). *C*, dehydroascorbic acid uptake in the presence (○) or in the absence (●) of NaCl. CaCo-2 cells were plated in 12-well plates, and uptake was measured at 24 h. Uptake experiments were performed at 37 °C using 50 μM AA or DHA. Data represent the mean $\pm$ S.D. of experiments performed in triplicate.
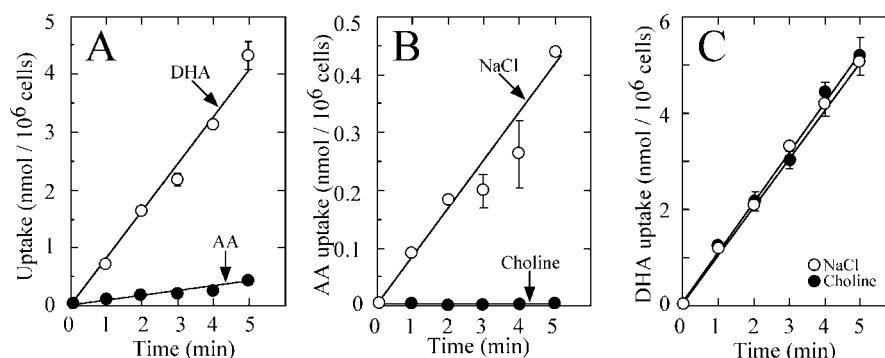
(810 pmol/min per 10^6 cells) or absence (805 pmol/min per 10^6 cells) of NaCl (Fig. 1*C*).

A time course analysis of dehydroascorbic acid uptake using substrate concentrations from 50 μM to 30 mM revealed that the uptake rate was constant for at least 3 min at each tested concentration, indicating that these values represent real transport rates (Fig. 2*A*). A study of dehydroascorbic acid transport dependence on the substrate concentration showed that the rate of transport approached saturation at 20 mM dehydroascorbic acid (Fig. 2*B*). Transport data analysis using the Eadie-Hofstee method revealed the presence of at least two different functional components, involved in dehydroascorbic acid transport by CaCo-2 cells, each having different affinities for the substrate (Fig. 2*C*). The higher affinity component had an apparent K_m of 0.7 mM and a V_{max} of 15 nmol/min per 10^6 cells for the transport of dehydroascorbic acid. In comparison, the lower affinity component had an apparent transport K_m of 4.5 mM and a V_{max} of 30 nmol/min per 10^6 cells (Fig. 2*C*). Competition and inhibition experiments revealed that 50 mM deoxyglucose decreased dehydroascorbic acid transport by more than 90%, whereas 50 mM sucrose, 50 mM fructose, or 10 mM α-methyl-D-glucoside had no effect. Furthermore, 20 μM cytochalasin B, but not cytochalasin E, inhibited transport by more than 85% (Fig. 2*D*). In parallel experiments, we studied the transport of deoxyglucose. Time course analysis using deoxyglucose concentrations from 1 to 40 mM defined a temporal window of 1 min for the transport assays (Fig. 2*E*). Dose-response analysis (Fig. 2, *F–G*) revealed the presence of at least two different kinetic components involved in deoxyglucose transport by CaCo-2 cells. The higher affinity component had apparent transport K_m and V_{max} values of 0.9 mM and 15 nmol/min per 10^6 cells, respectively. The lower affinity component had K_m and V_{max} values of 23 mM and 90 nmol/min per 10^6 cells, respectively. The transport and kinetic data are compatible with expression of the transporters GLUT3 and GLUT2 in CaCo-2 cells. Expression of GLUT3 and GLUT2 was confirmed by RT-PCR analysis using primers specific for the different transporter isoforms and by immunolocalization experiments using antibodies specific for the glucose transporter isoforms GLUT1 to GLUT5 (data not shown). These experiments also revealed that CaCo-2 cells express GLUT1, a transporter of intermediate affinity, and a closer analysis of the deoxyglucose transport data reveals the possible presence of a third functional component (Fig. 2*G*) that would be consistent with the presence of GLUT1 in these cells. No such third component is evident from the dehydroascorbic transport data (Fig. 2*C*), which is consistent with the similar transport K_m of GLUT1 and GLUT3.

Next we examined the characteristics of ascorbic acid transport in CaCo-2 cells based on the initial evidence of its sodium dependence. Uptake proceeded in a linear fashion for at least 6 min at a substrate concentration of 10–500 μM (Fig. 3*A*). A

detailed dose-response study showed that the transport of ascorbic acid approached saturation at about 500 μM substrate (Fig. 3*B*). Analysis of the transport data by the Eadie-Hofstee method revealed the presence of at least two different functional components, involved in ascorbic acid transport by CaCo-2 cells, each with different affinities for the substrate (Fig. 3*C*). The higher affinity component had an apparent transport K_m of 8 μM and a V_{max} of 40 pmol/min per 10^6 cells, whereas the lower affinity component had apparent K_m and V_{max} values of 125 μM and 670 pmol/min per 10^6 cells, respectively. Analysis of the sodium effect on ascorbic acid transport at 10 μM, conditions at which greater than 70% of the total transport is expected to be mediated by the higher affinity transporter, revealed that this transporter was strongly activated by sodium (Fig. 3*D*). The sodium effect was of a cooperative nature as indicated by the sigmoidal shape of the dose-dependent curve, a conclusion that was corroborated when the transport data were utilized to construct a Hill plot that resulted in a straight line having a Hill coefficient (the slope of the Hill graph) of 1.8 (Fig. 3*F*). Similar results were obtained when using 500 μM ascorbic acid, a concentration at which it is expected that greater than 70% of the total transport would be mediated by the lower affinity transporter. The dose-dependent curve was sigmoidal (Fig. 3*E*) with a Hill coefficient of 1.9 (Fig. 3*F*). Competition and inhibition experiments revealed that deoxyglucose, sucrose, fructose, and cytochalasin B failed to affect ascorbic acid transport, indicating that the ascorbic acid transporters expressed by CaCo-2 cells are functionally unrelated to the dehydroascorbic acid transporters.

We performed RT-PCR with primers specific for SVCT1 and SVCT2, the two human isoforms currently cloned, in order to confirm the existence of two sodium-dependent ascorbic acid transporters in CaCo-2 cells, as well as to identify them. RT-PCR experiments yielded amplification products having the expected sizes for SVCT1 (304 bp) and SVCT2 (459 or 307 bp) (Fig. 4 and data not shown). The specificity of the amplification reaction was verified by performing the RT-PCR assays using human brain for SVCT2 (Fig. 4) and human intestinal cells for SVCT1 (data not shown) as the RNA sources. PCR amplification products were isolated and submitted to automated sequencing, which confirmed their identities as corresponding to products amplified from the coding sequences of SVCT1 and SVCT2.

When cultured *in vitro* for several days and allowed to reach confluence, CaCo-2 cells underwent further differentiation along the enterocyte pathway which is accompanied by changes in the expression of proteins that are considered markers of terminal differentiation. CaCo-2 cells cultured for 15 days reached a post-confluence state that was characterized by a notable increase in their capacity to transport ascorbic acid. Transport studies using 10 and 500 μM ascorbic acid revealed that the increase in transport was fundamentally associated
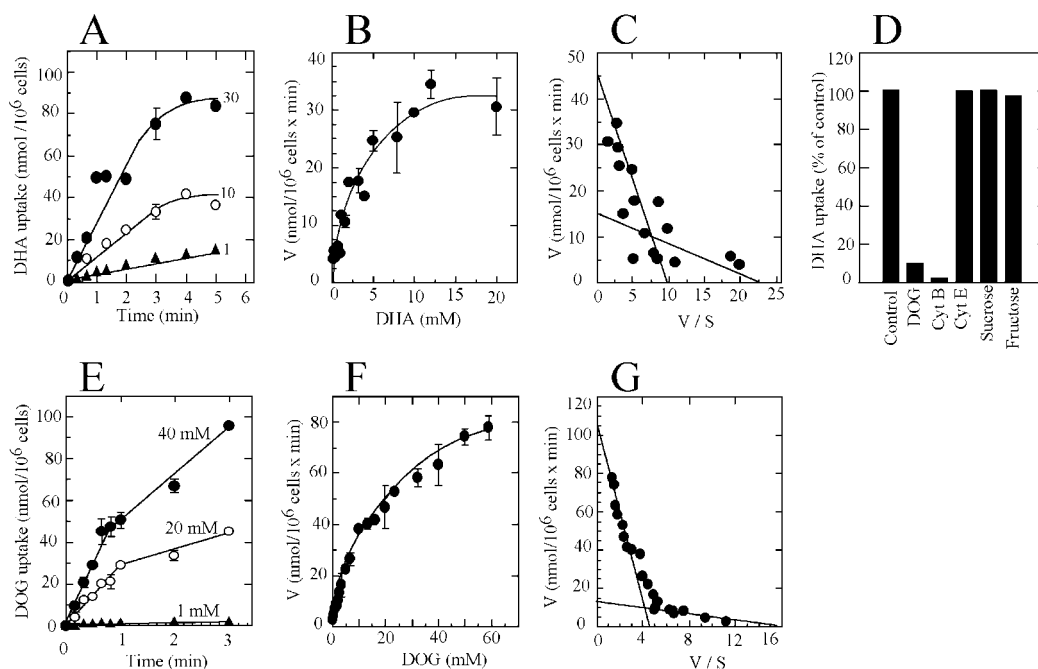
FIG. 2. **Dehydroascorbic acid and deoxyglucose transport in CaCo-2 cells.** *A*, time course of 1 (▲), 10 (○), or 30 mM (●) DHA uptake. *B*, dose-response analysis of DHA transport. *C*, Eadie-Hofstee plot of substrate dependence for DHA transport. *D*, effect of glucose transporter substrates (2-deoxy-D-glucose, sucrose, and fructose) and inhibitors (cytochalasins B and E) on DHA transport. Transport in the presence of the test compounds is expressed as percent of control (transport in the absence of substrates or inhibitors). *E*, time course of 1 (▲), 20 (○), or 40 mM (●) 2-deoxy-D-glucose (*DOG*) uptake. *F*, dose-response analysis of 2-deoxy-D-glucose transport. *G*, Eadie-Hofstee plot of substrate dependence for 2-deoxy-D-glucose transport. CaCo-2 cells were plated in 12-well plates, and uptake of DHA or 2-deoxy-D-glucose was measured at 24 h. Uptake experiments were performed at room temperature. Data represent the mean ± S.D. of experiments performed in triplicate.

FIG. 3. **Ascorbic acid transport in CaCo-2 cells.** *A*, time course of 10 (▲), 50 (○), or 500 μM (●) AA uptake. *B*, dose-response analysis of AA transport. *C*, Eadie-Hofstee plot of the substrate dependence for AA transport. *D*, effect of sodium on transport of 10 μM AA. *E*, effect of sodium on transport of 500 μM AA. *F*, Hill plots for the sodium effect on transport of 10 (●) or 500 μM (○) AA. CaCo-2 cells were plated in 12-well plates, and uptake was measured at 24 h. Uptake experiments were performed at 37 °C. Data represent the mean ± S.D. of experiments performed in triplicate.
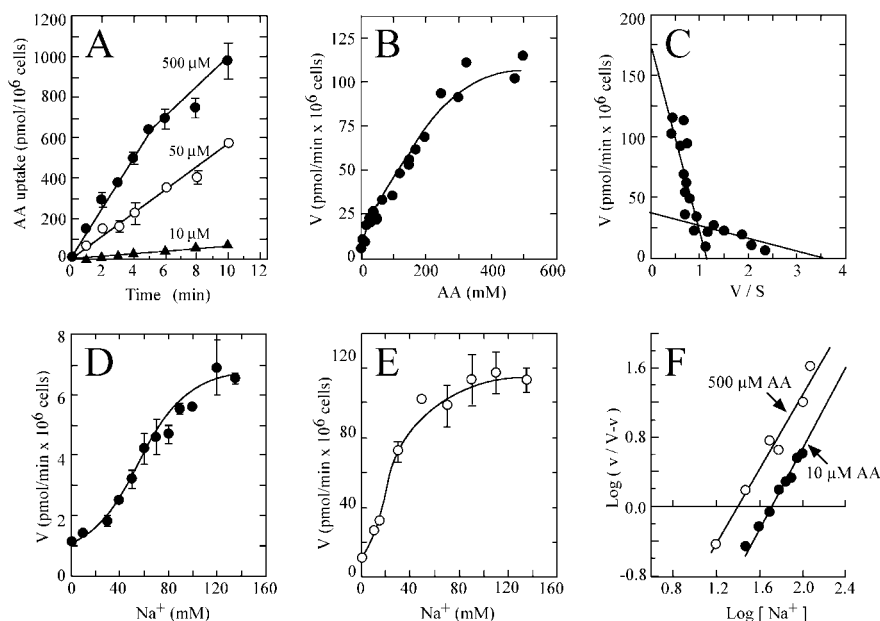
with the increased expression of the lower affinity component. At 10 μM ascorbic acid, the transport rate first increased from 11.5 pmol/min per 10^6 cells at day 2 post-seeding (pre-confluent cells) to 15.5 pmol/min per 10^6 cells at day 6, and then decreased to 9 pmol/min per 10^6 cells at day 10 to reach a lower value of 5 pmol/min per 10^6 cells at day 15 (Fig. 5, *A* and *B*). In contrast, the transport rate at 500 μM ascorbic acid increased from 70 pmol/min per 10^6 cells at day 2 to 160 pmol/min per 10^6 cells at day 6 and reached a value of 250 pmol/min per 10^6 cells at day 15 (Fig. 5, *C* and *D*). Dose-response analysis using increasing concentrations of ascorbic acid revealed that the transport rate at saturation was about 2-fold higher in post-

confluent (15-day culture) as compared with pre-confluent (2-day culture) cells (Fig. 5*E*). Analysis of the transport data by the Eadie-Hofstee method confirmed the previous observation by revealing a 1.7-fold increase in the V_{max} of transport in 15-day-old cells compared with those at 2 days (Fig. 5*F*). Moreover, whereas two functional components were present in 2-day-old cells, only the lower affinity component was present at 15 days. At 2 days, the higher affinity component had apparent K_m and V_{max} values of 8 μM and 40 pmol/min per 10^6 cells, respectively, whereas the lower affinity component had K_m and V_{max} values of 125 μM and 80 pmol/min per 10^6 cells, respectively. In contrast, 15-day-old cells displayed a lower

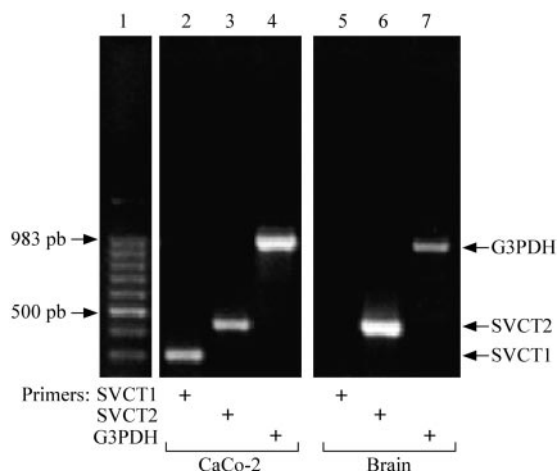FIG. 4. **RT-PCR analysis of SVCT1 and SVCT2 expression in CaCo-2 cells.** PCR analysis was performed using CaCo-2 cDNA (*lanes 2–4*) or total human brain cDNA (*lanes 5–7*) as templates with specific primers for SVCT1 (*lanes 2* and *5*) or SVCT2 (*lanes 3* and *6*). Amplification of glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) (*lanes 4* and *7*) was used as an internal control. The products were separated on 1.5% agarose gel and stained with ethidium bromide. DNA bands in *lane 1* correspond to size markers.

affinity component with apparent K_m and V_{max} values of 110 μM and 180 pmol/min per 10^6 cells, respectively.

We interpreted our transport data as indicating that the post-confluent differentiation of CaCo-2 cells is accompanied by a marked increase in the expression of functional SVCT1 transporters, changes that occurred independently of any change in SVCT2 expression. We further analyzed this issue using RT-PCR with primers specific for SVCT1 and SVCT2 and RNA prepared from pre- and post-confluent cells. These experiments revealed that post-confluence was associated with an increase in the steady-state SVCT1 mRNA levels, as indicated by a marked increase in the intensity of the amplification product without detectable changes in SVCT2 mRNA (data not shown). To confirm these findings, we performed quantitative RT-PCR for SVCT1 and SVCT2 using RNA isolated from CaCo-2 cells maintained in culture from 2 to 20 days. We used changes in GLUT1, GLUT2, and GLUT5 RNA as markers for post-conflu-ent CaCo-2 cell differentiation (25, 26) with β-actin as an internal control. A standard curve was constructed optimizing the PCR for each transporter and for β-actin, and the respec-tive cross-over points were used for quantification. The control experiments revealed that GLUT1 and GLUT5 were initially present in CaCo-2 cells at equivalent levels of expression for the first 4 days of culture. However, post-confluent differenti-ation of the CaCo-2 cells was accompanied by a marked in-crease in the amount of GLUT5 mRNA. At day 15, the amount of GLUT5 mRNA was ~8-fold greater than at day 1 (Fig. 6*A*). Further control experiments revealed that β-actin expression was maintained at constant levels during the duration of the experiment (Fig. 6*A*). Parallel experiments revealed that SVCT1 and SVCT2 are independently regulated during the post-confluent differentiation of the CaCo-2 cells (Fig. 6*B*). SVCT1 levels increased as differentiation proceeded, and at day 20 there was 5-fold more SVCT1 mRNA than at day 2. On the other hand, there was no increase in SVCT2 mRNA levels during differentiation, confirming the results of the functional studies indicating that SVCT1 was selectively up-regulated during post-confluent differentiation of the CaCo-2 cells.

When CaCo-2 cells were cultured in permeable filter inserts (transwell system), they formed an impermeable barrier con-sisting of highly polarized cells tightly packed and with the establishment of tight junctions. Under these conditions, the
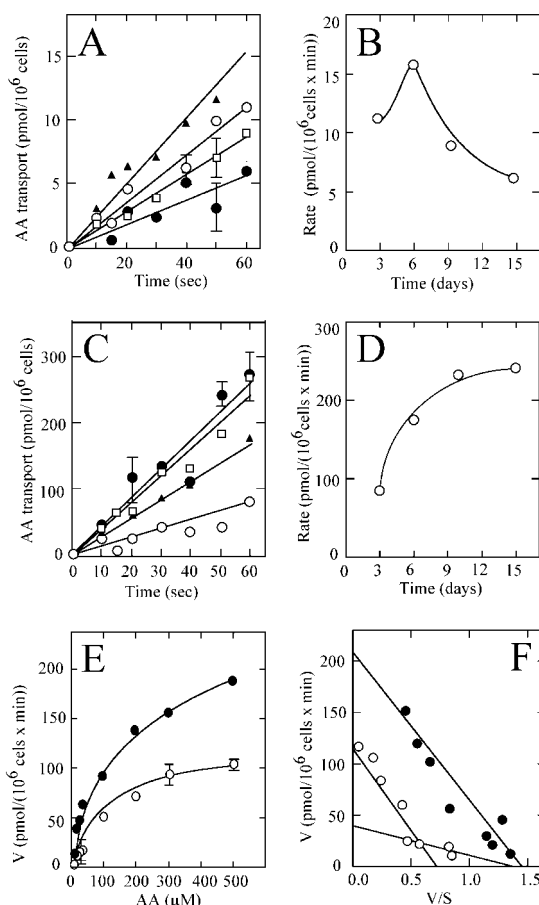

FIG. 5. **Ascorbic acid transport in pre-and post-confluent CaCo-2 cells.** *A*, transport of 10 μM AA in CaCo-2 cells cultured for 2 (○), 6 (▲), 10 (□), and 15 (●) days. *B*, rate of AA transport from data in *A*. *C*, transport of 500 μM AA in CaCo-2 cells cultured for 2 (○), 6 (▲), 10 (□), and 15 (●) days. *D*, rate of AA transport derived from data in *C*. *E*, dose-response analysis of AA transport in CaCo-2 cells cultured 3 (○) or 15 (●) days. *F*, Eadie-Hofstee plot of the substrate dependence for AA transport in 3- (○) or 15-day (●) cells. Transport was measured at 37 °C. Data represent the mean ± S.D. of experiments performed in triplicate.
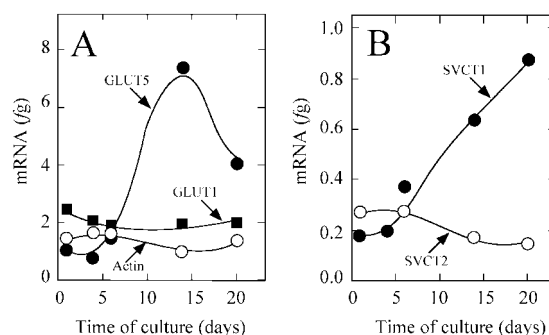

FIG. 6. **Quantitative analysis of hexose and ascorbic acid transporter expression in post-confluent CaCo-2 cells.** *A*, quan-titative RT-PCR analysis of GLUT1 (○), GLUT5 (●), and actin (■) mRNA expression in CaCo-2 cells grown to confluence. CaCo-2 cells were cultured in 6-well plates, and RNA was prepared at days 1, 4, 6, 14, and 20 after seeding and used to synthesize the corresponding cDNAs. Expression of the test proteins was assessed by quantitative PCR using specific primers and real time PCR with the Light Cycler™. *B*, quantitative RT-PCR analysis of SVCT1 (●) and SVCT2 (○) mRNA expression in CaCo-2 cells grown to confluence. Details are the same as in *A*.

culture medium present in the upper compartment of the tr-answell system was in contact only with the apical region of the cells and did not contact the basolateral plasma membrane,
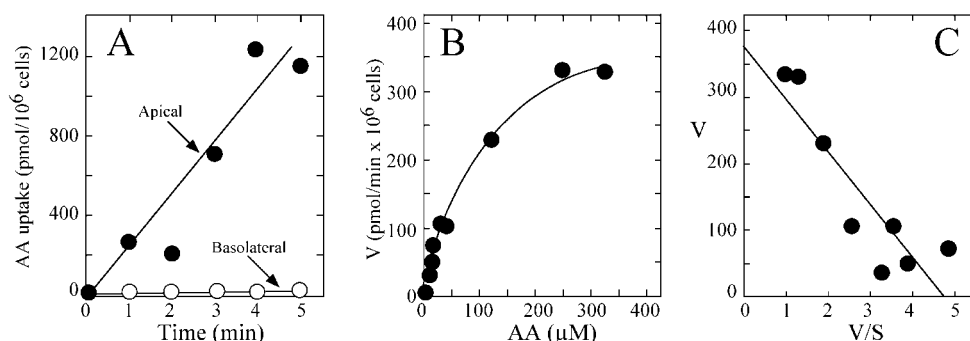
Vitamin C Transporters in CaCo-2 Cells

FIG. 7. **Ascorbic acid transport in polarized CaCo-2 cells.** *A*, transport of AA in polarized CaCo-2 cells cultured in two-chamber permeable membrane inserts. CaCo-2 cells were seeded on collagen-coated transwell inserts in 6-well plates and allowed to grow for 15 days. Uptake of AA was measured from the apical (●) or basolateral (○) transwell compartment. *B*, dose-response analysis of apical AA transport in 15-day cells. *C*, Eadie-Hofstee plot of the substrate dependence for apical AA transport in 15-day cells. Transport was measured at 37 °C. Data represent the mean of two independent experiments.

whereas the culture medium present in the lower compartment was in contact with the basolateral region of the cell and did not contact the apical plasma membrane. We hypothesized whether the post-confluent differentiation of CaCo-2 cells growing in transwells was accompanied not only by an increased expression of SVCT1 but also by the specific sorting of the transporters to the apical or to the basolateral membrane compartments. The transport experiments revealed that there was no transport of reduced vitamin C through the basolateral plasma membrane and that ascorbic acid was exclusively taken up through the apical plasma membrane (Fig. 7A). Kinetic analysis of ascorbic acid transport revealed that transport approached saturation at about 400 μM substrate (Fig. 7B), and analysis of the data by the Eadie-Hofstee transformation revealed that the transport of ascorbic acid at the apical membrane compartment was mediated by a single component with an apparent K_m of 105 μM and an apparent V_{max} of 350 pmol/min $\times$ 10^6 cells (Fig. 7, *B* and *C*). Overall, our data can be interpreted as indicating that SVCT1 is selectively expressed and sorted to the apical plasma membrane of the CaCo-2 cells during differentiation and polarization.

DISCUSSION

The human colon carcinoma cell line CaCo-2 is a valuable cell system to model the mechanism and regulation of nutrient transport in human intestinal epithelium (30, 31, 33). Therefore, we selected this cell system to perform a detailed characterization of vitamin C transport in enterocyte-like cells. We concluded that CaCo-2 cells acquire both forms of vitamin C, the oxidized form (dehydroascorbic acid) and the reduced form (ascorbic acid). CaCo-2 cells acquire oxidized vitamin C with the participation of two, and perhaps three different transporter activities. The fact that sodium was not required for transport discards the participation of a sodium-glucose co-transporter (SGLT) in this process. Results of competition experiments, showing that deoxyglucose, but not α-methyl-D-glucoside, blocked the transport of dehydroascorbic acid, and the strong inhibitory effect of cytochalasin B indicated that dehydroascorbic acid transporters expressed in CaCo-2 cells correspond to facilitative glucose transporters. This conclusion is consistent with previous evidence indicating that CaCo-2 cells express facilitative glucose transporters (GLUTs) and that oxidized vitamin C is transported exclusively by members of this transporter family (2, 14, 16, 17). RT-PCR corroborated the expression of GLUT1, GLUT2, GLUT3, and GLUT5 in CaCo-2 cells. GLUT5 is a fructose transporter that does not transport glucose or dehydroascorbic acid. On the other hand, apparent K_m values for the transport of dehydroascorbic acid and deoxyglucose suggest that the higher affinity transporter present in

CaCo-2 cells corresponds to GLUT3. Although GLUT1 is expressed in CaCo-2 cells, its transport K_m for both dehydroascorbic acid and deoxyglucose is similar to that of GLUT3 and cannot be differentiated from this transporter using functional assays. A third, low affinity dehydroascorbic acid transporter detected in CaCo-2 cells was identified as GLUT2 based on transport and RT-PCR analysis. Although the ability of GLUT2 to transport dehydroascorbic acid is still under review, we have obtained evidence from studies using primary rat hepatocytes indicating that GLUT2 is a low affinity, high capacity transporter of dehydroascorbic acid.[2]

We also determined the presence of two Na$^+$-ascorbic acid co-transporters in CaCo-2 cells with different affinities for ascorbic acid transport. The lower affinity component had an apparent K_m of 125 μM, which is similar to the K_m described for the cloned human SVCT1 expressed in *Xenopus laevis* oocytes. Based on K_m data and RT-PCR results, we propose that the low affinity ascorbic acid transporter expressed in CaCo-2 cells is SVCT1. This is consistent with the fact that SVCT1 was cloned from a CaCo-2 cell cDNA library (20). The second higher affinity transporter of ascorbic acid detected in CaCo-2 cells had an apparent transport K_m of 8 μM, which is similar to that described for the cloned human SVCT2 transporter (21) expressed in *Xenopus* oocytes. RT-PCR analysis confirmed the expression of SVCT2 in CaCo-2 cells, supporting the concept that it corresponds to the higher affinity transporter of ascorbic acid present in these cells.

A central finding of this study is the evidence, obtained from functional assays coupled to quantitative RT-PCR analysis, indicating that SVCT1 and SVCT2 expressions are independently regulated in CaCo-2 cells during cell differentiation. Cells cultured for long periods showed a severalfold increase in SVCT1 mRNA expression without appreciable changes in SVCT2 mRNA levels. By using GLUT5 expression as a differentiation marker, we confirmed that SVCT1 mRNA expression was up-regulated coincidentally with the maturation of post-confluent cells. The net result is an increase in the amount of functional SVCT1 protein present at the plasma membrane as revealed by a marked increase in ascorbic acid transport. Furthermore, post-confluent CaCo-2 cells growing on permeable filter inserts showed selective sorting of SVCT1 to the apical membrane compartment. This is similar to the glucose transporter pattern developed by CaCo-2 cells grown under conditions that led to the expression of enterocyte-like properties, with polarized distribution of glucose transporters at the apical and/or basolateral surface of the cell, whereas the Na$^+$-depend-

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[2] J. G. Cárcamo and J. C. Vera, unpublished results.

ent transporter SGLT1 displays a unique subcellular distribution in the apical membrane compartment (24, 30, 40). In the normal absorptive epithelia of the human small intestine, glucose uptake from the lumen of the gut is mediated by SGLT1, and glucose efflux from these cells into the blood is mediated by GLUT2. Thus, both CaCo-2 cells and normal human enterocytes are characterized by the differential subcellular distribution of SGLT and GLUTs, which is a key element in the vectorial transfer of sugars from the intestinal lumen to the blood. In this context, our data open up the possibility that the polarized distribution of vitamin C transporters at the apical or basolateral membrane compartment of the intestinal enterocytes may play an important role in the vectorial transport of vitamin C across the intestinal barrier.

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**Up-regulation and Polarized Expression of
the Sodium-Ascorbic Acid Transporter
SVCT1 in Post-confluent Differentiated
CaCo-2 Cells**

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