Glyoxalate reductase/hydroxy pyruvate reductase interacts with the sodium-dependent vitamin C transporter-1 to regulate cellular vitamin C homeostasis

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VITAMIN C (ascorbic acid, AA) is an effective antioxidant that acts as a free radical scavenger and an essential cofactor for many enzymatic reactions (26). Although many mammals can synthesize vitamin C via the glucuronic acid pathway in the liver, humans have lost the capability for endogenous vitamin C synthesis during the course of evolution and must obtain it from dietary sources. Several studies have shown that optimal vitamin C body homeostasis protects against gallbladder and cardiovascular disease, non alcoholic steatohepatitis, eye disorders, and cancer (15, 19, 33). Deficiency of dietary vitamin C can cause a variety of clinical abnormalities including scurvy, delayed wound healing, bone and connective tissue damage, as well as vasomotor instability (7, 19, 26). Conversely, excessive vitamin C intake may cause hyperoxaluria and kidney stone formation, since oxalate is an end product of AA catabolism (16, 25, 30, 39).

The mechanism of AA uptake by mammalian cells is Na+ and temperature dependent and occurs via specialized carrier-mediated system(s) (28, 29, 32). Two vitamin C transporters have been cloned in humans, the sodium-dependent vitamin C transporter-1 and -2 (hSVCT1 and hSVCT2), the products of SLC23A1 and SLC23A2 genes (11, 27, 42, 43). Each transporter possesses a 12-transmembrane spanning topology with cytoplasmic NH2- and COOH-terminal domains and consensus motifs for posttranslational modification (20, 35). However, little is known about the broader hSVCT interactome since no regulatory partners have been described to date. This contrasts with many other water-soluble vitamin transporters for which regulatory protein partners have been identified (1, 23, 24).

To identify proteins that interact with hSVCT1, we employed a yeast two-hybrid (Y2H) screening approach in a human liver cDNA library, from which we identified human glyoxalate reductase/hydroxy pyruvate reductase (hGR/HPR) as an associated partner of hSVCT1. hGR/HPR is a predominantly hepatic enzyme that has a dual enzymatic activity: converting the metabolic by-product glycolate to glycolate and hydroxy pyruvate to n-glyceral. The reduction of glyoxylate to glycylate by hGR/HPR mitigates a competing oxidation of glyoxylate to oxalate and a subsequent risk of oxalate toxicities (“hyperoxaluria”). Indeed, several different mutations in hGR/HPR underpin type 2 primary hyperoxaluria (PH2 [8, 18, 38]), a rare autosomal recessive disorder characterized by overproduction of oxalate. The increased plasma and urinary oxalate levels resulting from hGR/HPR loss of function drive the deposition of calcium oxalate and may lead to urolithiasis and nephrocalcinosis. Therefore, the foundational information from the Y2H screen that hGR/HPR (decreases cellular oxalate) and hSVCT1 (elevates cellular oxalate as result of ascorbate catabolism) may interact structurally was intriguing. Subsequent experiments to validate and assess the significance of this interaction are described in this study.

MATERIALS AND METHODS

Materials. [14C]AA (specific activity 13 mCi/mmol, radiochemical purity ≈98%) was obtained from American Radiolabeled Chemical (St. Louis, MO). HepG2, HuTu-80, HK-2, Caco-2, and ARPE-19 cells were from ATCC (Manassas, VA) and NCM460 cells were obtained from INCELL (San Antonio, TX) (31). DNA oligonucleotide primers were synthesized by Sigma Genosys (Woodlands, TX). Genetecin (G418) was from Invitrogen (Carlsbad, CA). All chemicals and reagents used in this study were of analytical/molecular biology grade.

Cell culture; transient and stable transfections. The human-derived liver HepG2, duodenal HuTu-80, kidney HK-2, intestinal Caco-2, and...
retinal pigment ARPE-19 epithelial cells were grown in DMEM, and normal corneal epithelial NCM460 cells were grown in Ham’s F-12 culture medium supplemented with 10 or 20% (vol/vol) fetal bovine serum, glutamine (0.29 g/l), sodium bicarbonate (2.2 g/l), penicillin (100,000 U/l), and streptomycin (10 m g/l) in 75-cm² plastic flasks at 37°C in a 5% CO₂-95% air atmosphere with media changes every 2–3 days. For transient transfection, cells were grown on sterile 12-well plates (Corning, NY) or glass-bottomed Petri dishes (MatTek, MA) and transfected at 90% confluency with 4-µg plasmid DNA by use of Lipofectamine 2000 (Invitrogen). After 24–48 h, cells were used for uptake assays, mRNA analysis or live cells were imaged by confocal microscopy. For stable transfection, HepG2 cells were selected by using G418 (0.5 mg/ml) for 6–8 wk as described previously (34, 35).

**Y2H and 1-by-1 Y2H assays.** Y2H and 1-by-1 Y2H interaction were performed by a commercial service (Hybrigenics, Paris, France) following previously described procedures (13). Briefly, the hSVCT1 COOH-terminal (511–598 amino acids) was PCR-amplified and cloned into pB2 as a COOH-terminal fusion to LexA (N-LexA-hSVCT1-C) and used as a bait to screen a random-primated human liver cDNA library constructed into pB6 plasmid. The pB27 and pB6 plasmids were derived from pBTM116 (41) and pGADGH (3) plasmids, respectively. Eighty-one million colonies (8-fold the complexity of the library) were screened via a mating approach with Y187 (mate) and L40Gal4 (mate) yeast strains as described previously (13). The prey fragments of positive clones were amplified by PCR and sequenced. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) and a confidence score (predicted biological score) attributed to each potential interaction (12). For 1-by-1 Y2H interaction analysis, the COOH-terminal of hSVCT1 was PCR amplified and cloned in frame with the LexA DNA binding domain (DBD) into pB27 and the fragment corresponding to 79–280 amino acids of hGR/HPR protein was isolated from the human liver cDNA library. It was cloned in frame with the Gal4 activation domain (AD) into plasmid pB6. Both DBD and AD constructs were verified by sequencing. The interaction between hSVCT1 and hGR/HPR was determined on the basis of the activity of the reporter gene HIS3 (growth assay without histidine).

**GST pull-down assay.** The full-length glutathione-S-transferase (GST)-hGR/HPR recombinant protein was obtained from Abnova (Walnut, CA). The hSVCT1 stably expressing HepG2 cells were lysed in Celllytic M cell lysis reagent (Sigma) and the cleared post nuclear extract was obtained by centrifugation (14,000 rpm for 10 min at 4°C). The GST-hGR/HPR and GST proteins (100 µg) were preincubated with glutathione-Sepharose 4B beads (GE Healthcare, Piscataway, NJ) and washed with cell lysis buffer. The cleared postnuclear extract of HepG2 cells (1 mg of total soluble protein) was incubated either with GST-hGR/HPR or GST bound to glutathione-Sepharose 4B beads (GE Healthcare) in Celllytic M cell lysis reagent (Sigma) for 2 h at 4°C. Proteins bound to beads were eluted with 10 mM glutathione and analyzed by Western blotting using either a rabbit polyclonal or monoclonal anti-hSVCT1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA/Abgent, San Diego, CA). The immunoreactive bands were detected with anti-rabbit IRDye-800 or anti-mouse IRDye-680 secondary antibodies (LI-COR Bioscience, Lincoln, NE) by use of an Odyssey infrared imaging system (LI-COR Bioscience) (36, 37).

**Coimmunoprecipitation assays.** HepG2 cells transiently transfected with pFLAG-CMV-2-hGRHPR and parallel nontransfected control cells were used in coimmunoprecipitation studies (1, 23, 24). Briefly, cells were lysed in RIPA cell lysis reagent (Sigma) with complete protease inhibitor cocktail (Roche Applied Sciences) and centrifuged (14,000 rpm for 20 min at 4°C). Cleared lysates were then incubated with anti-FLAG M2 antibody conjugated beads (Sigma) for 3 h at 4°C in rotating wheels. Immunoprecipitated proteins and total cell lysate samples were then separated on NuPAGE 4–12% Bis-Tris mini gels (Invitrogen) and analyzed by Western blotting as described above. FLAG-tagged hGR/HPR was detected with a monoclonal anti-FLAG M2 antibody (Sigma). Mammalian two-hybrid assay. A mammalian two-hybrid system (CheckMate, Promega, Madison, WI) was used as per manufacturer’s instructions. The full-length coding sequences for hSVCT1 and hGR/HPR were cloned in frame into pBIND and pACT vectors by using gene specific primers (Table 1) by PCR amplification followed by ligation and transformation into Escherichia coli (JM109) cells. The pACT-hGR/HPR and pBIND-hSVCT1 constructs (4 µg each) were cotransfected into HepG2 cells to generate hSVCT1 fused with the DNA-binding domain of GAL4 and hGR/HPR fused with the activation domain of VP16, respectively. The pG5Luc vector, containing the firefly luciferase gene under the control of a transcriptional unit comprising five GAL4-binding elements upstream of a minimal TATA box, served as a reporter gene. Renilla-normalized firefly luciferase activity was assayed after 72 h of transfection utilizing a dual-luciferase assay system (Promega) and luminometer (Turner Designs Instruments, Sunnyvale, CA).

**Confocal microscopy.** Human-derived liver HepG2 cells were grown on glass-bottomed Petri dishes (MatTek, Ashland, MA) and transfected with GFP-hSVCT1 and hGR/HPR-mCherry constructs (4 µg) at 90% confluency using 4 µl of Lipofectamine 2000 (Invitrogen). Live cell confocal imaging was performed 24–48 h following transfection. The floresphores were excited by using 488 nm (GFP) and 568 nm laser lines and emitted fluorescence was monitored with a 515 ± 30-nm band-pass (GFP) or at 630 ± 60-nm long-pass (mCherry) filter.

**siRNA analysis.** Small interfering RNA (siRNA) for hGR/HPR was obtained from Santa Cruz Biotechnology. ARPE-19 cells (90% confluent) were transiently transfected with siRNA [pool of three different siRNA duplexes (duplex-1, sense: CCAGAUGUCCUCAACA-AU-A, antisense: UAUCUGACGUACUUGGt; duplex-2, sense: CUCUUGUUGCAGCUAACA-Att, antisense: UUGUAGUGUCCCAACAGGt; duplex-3, sense: GGAAGAGUGUACUUGGt].

**Gene Name** | **Forward and Reverse Primers (5’-3’)**
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Mammalian two hybrid assay | hSVCT1: GCTTTGGACATTATGAGGCCCCAGAGACG; GCCCATGAAGAGCCATCCCTAGCAAAATAGACGTTCAACACTTGATCGATTC | hGR/HPR: TCCCTGACAGACCTGTGGCAGGCAAGACGGTCTCCAGCCGACG
Coimmunoprecipitation | hSVCT1: CCAAGCAGTATAGGAGGCCCCAGAGACG; GCCCATGAAGAGCCATCCCTAGCAAAATAGACGTTCAACACTTGATCGATTC | hGR/HPR: TCCCTGACAGACCTGTGGCAGGCAAGACGGTCTCCAGCCGACG
Confocal studies | hSVCT1: CCAAGCAGTATAGGAGGCCCCAGAGACG; GCCCATGAAGAGCCATCCCTAGCAAAATAGACGTTCAACACTTGATCGATTC | hGR/HPR: TCCCTGACAGACCTGTGGCAGGCAAGACGGTCTCCAGCCGACG
Real-time PCR studies | hSVCT1: TATCATCCTCTCTCCTACATCT; AGAAGACCGCAACAGGTCAT | hGR/HPR: TATCATCCTCTCTCCTACATCT; AGAAGACCGCAACAGGTCAT

Table 1 shows the primer sequence and combination of primers used to generate each hSVCT1 and hGR/HPR construct by PCR. Restriction sites for HindIII (boldface text), SacII (boldface underlined text), XhoI (boldface italics), KpnI (italics), and Sulf (underlined text) were added to the hSVCT1 and hGR/HPR primers to allow subsequent subcloning into the GFP-C1, pFLAG, mCherry, pBIND, and pACT vectors.
GAUAtt. antisense: UAUCAGAUAUCACUCUUUUCU(T) specific for hGR/HPR and negative control siRNA (scrambled) by use of Lipofectamine 2000. After 48 h, total RNA was isolated from these cells to determine hSVCT1, hSVTCT2, and hGR/HPR mRNA expression levels and to assess the effect of hGR/HPR silencing on [14C]AA uptake.

Real-time PCR. Total RNA (5 μg) isolated from cell lines was treated with DNase I and subjected to reverse transcription with Superscript II kit (Invitrogen). The products were then used for real-time PCR amplification with hSVCT1, hSVTCT2, hGR/HPR and human β-actin primers (Table 1). Data were normalized to simultaneously amplified human β-actin, and cycle threshold values were calculated by the relative relationship method (21).

Uptake studies. [14C]AA uptake was performed at 37°C on confluent HepG2 or ARPE-19 cells (48 h after transfection) in Krebs-Ringer buffer (28, 35). The labeled [14C]AA was added to the incubation buffer at the onset of incubation, and uptake quantification was performed during the initial linear period (3 min). The reaction was terminated by the addition of ice-cold buffer, and radioactivity and protein contents were determined (28, 35). Uptake data are the results of at least three independent experiments and are expressed as means ± SE (percentage over control). Statistical analysis was performed by the Student’s t-test with statistical significance level being set at P < 0.05.

RESULTS

hGR/HPR interacts with hSVCT1 COOH-terminal in a Y2H analysis. Recent truncation/mutational studies have shown that the COOH-terminal sequence of hSVCT1 is important for transporter function and targeting to cell membrane (34). To identify proteins interacting with this region of hSVCT1, the cytoplasmic COOH-terminal domain of hSVCT1 (amino acids 511–598) was used as bait in a Y2H screen (Fig. 1A). This construct (as a LexA fusion) was assayed against prey in a random-primed human liver cDNA library. From this screen, His+ colonies were selected and the prey fragments of the positive clones were amplified by PCR and sequenced. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI). The positive clone with the highest confidence score for interaction was confirmed to be hGR/HPR, an enzyme predominantly expressed in liver and kidney. A subsequent 1-by-1 Y2H interaction assay was performed using the DNA binding domain, N-LexA-hSVCT1-C as bait and a N-GAL4 activation domain-hGR/HPR construct (79–280 amino acids) as the prey clone. The results of the 1-by-1 interaction assay confirmed solid colony growth for the assayed hSVCT1-hGR/HPR interaction in higher yeast dilution and under restrictive growth conditions [selective medium lacking tryptophan, leucine, and histidine but containing 3-aminotriazole (10 mM, an inhibitor of the HIS3 gene product)] compared with the positive and negative controls (Fig. 1B). These data implicate hGR/HPR as an interacting partner with the hSVCT1 COOH-terminal region.

Confirmation of hGR/HPR interaction with hSVCT1 in HepG2 cells. An in vitro GST pull-down assay was used to evaluate the interaction between hSVCT1 and hGR/HPR. An affinity-purified GST-hGR/HPR construct and GST alone were compared in their ability to pull down hSVCT1 from extracts prepared from hSVCT1-YFP stably expressing HepG2 cell line. These experiments revealed that GST-hGR/HPR, but not GST alone, recovered hSVCT1 from the cell extract (Fig. 2A). Next, we performed coimmunoprecipitation experiments to probe interactions between hGR/HPR and endogenous hSVCT1 in HepG2 cells. We transiently transfected pFLAG-hGR/HPR into HepG2 cells and incubated the isolated cell lysates with anti-FLAG M2 antibody-conjugated beads prior to probing immunoprecipitates by Western blotting. Results showed that...
the pFLAG-hGR/HPR immunoprecipitate contained endogenous hSVCT1. hSVCT1 was not detected in immunoprecipitates prepared from nontransfected HepG2 (control) cells (Fig. 2B). Anti-pFLAG-M2 antibodies confirmed the expression of hGR/HPR (37 kDa) protein in pFLAG-hGR/HPR transiently transfected HepG2 cell lysate (Fig. 2C). Finally, we performed a mammalian two-hybrid firefly luciferase assay in HepG2 cells. hSVCT1 and hGR/HPR were subcloned as GAL4 and VP16 fusion constructs in pBIND-hSVCT1 and pACT-hGR/HPR plasmids and coexpressed in HepG2 cells with a pG5 luc reporter vector. Renilla-normalized firefly luciferase activity was determined by use of a dual-luciferase assay system. Data are presented as means ± SE of at least 3 independent experiments and firefly luciferase expression was given in folds over the background (pACT/pBIND empty vectors) was set at 1. *P < 0.02.

Functional aspects of the interaction between hSVCT1 and hGR/HPR. On the basis of the above findings, we examined the effect of the hGR/HPR interaction with hSVCT1 on the function of hSVCT1 transporter. Uptake studies were performed by comparing [14C]AA uptake (32 μM, pH 7.4) in HepG2 cells overexpressing hSVCT1 alone and HepG2 cells coexpressing hSVCT1 and hGR/HPR. To do this, HepG2 cells stably expressing hSVCT1 were transiently cotransfected with the epitHELIAL cells. Results using hGR/HPR and hSVCT1 primers showed that all cells tested were positive for both hGR/HPR and hSVCT1 mRNA (Fig. 3, A and B), suggesting that both are likely coexpressed in absorptive epithelia. Because the liver is the predominant site of expression of hGR/HPR, we examined the extent of hSVCT1 and hGR/HPR colocalization in the HepG2 cell line. HepG2 cells were cotransfected with GFP-hSVCT1 and hGR/HPR-mCherry constructs and imaged 48 h after transfection. As expected, GFP-hSVCT1 displayed expression at the plasma membrane and also in numerous intracellular organelles as reported previously (28, 34, 35). hGR/HPR-mCherry expression was localized cytoplasmically and observed in association with a subset of hSVCT1 protein within intracellular organelles (Fig. 3C).
pFLAG-CMV-2-hGR/HPR and uptake was performed 24 h later. These data showed that coexpression of hSVCT1 with hGR/HPR resulted in a significant (P < 0.01) increase in [14C]AA uptake compared with cells expressing hSVCT1 alone (Fig. 4A). Reciprocally, we examined the effect of silencing the endogenous hGR/HPR gene using a siRNA approach. Owing to difficulties in knocking down endogenous hGR/HPR in HepG2 cells and lower expression of hSVCT1 (Fig. 3B), we used human retinal epithelial cells (ARPE-19) for these experiments. hGR/HPR is expressed in these cells (Fig. 3) and the cell line has been previously used to characterize hSVCT1 function (42). First, we assessed the specificity of hGR/HPR gene knockdown by performing real-time PCR. The real-time PCR results showed a significant (P < 0.01) decrease of endogenous hGR/HPR mRNA expression level in hGR/HPR siRNA-pretreated cells compared with scrambled siRNA-pretreated ARPE-19 cells. On the other hand, no change in hSVCT1 or hSVCT2 mRNA expression levels was detected in either hGR/HPR siRNA-pretreated cells or control cells (Fig. 4B). In parallel, uptake assays showed that knockdown of hGR/HPR significantly (P < 0.05) inhibited [14C]AA accumulation compared with ARPE-19 cells treated with scrambled siRNA alone (Fig. 4C). Therefore, overexpression of hGR/HPR increased cellular AA uptake, whereas knockdown of hGR/HPR impaired vitamin C accumulation.

DISCUSSION

Because the human liver plays an important role in vitamin C homeostasis, understanding the cellular and molecular mechanisms that regulate hepatic vitamin C uptake has significant physiological importance. Here, we identified hGR/HPR as a novel binding partner of hSVCT1 and show that the hGR/HPR-hSVCT1 interaction serves to influence cellular vitamin C accumulation. The hGR/HPR protein is a 328-amino acid enzyme (~37 kDa) that functions as a homodimer with a large coenzyme binding domain (107–298 amino acids) and a smaller substrate binding domains (5–106 and 299–328 amino acids) (4, 5). Under normal conditions hGR/HPR mediates the conversion of glyoxylate to glycolate in the liver. Deletion or mutation of the hGR/HPR gene results in increased amounts of glyoxylate and hydroxypyruvate, which are converted by lactate dehydrogenase to oxalate and L-glyceric acid. Overproduction of oxalate is accompanied by calcium oxalate deposition in various organs including the kidney, heart, and retina. As such, hGR/HPR deficiency causes primary hyperoxaluria type 2 (PH2), a disease associated with nephrolithiasis (calcium ox-
ate deposition in the renal pelvis and urinary tract), nephrocalcinosis (calcium oxalate deposition in the kidney parenchyma), and end-stage renal disease (8, 18, 38).

It is especially intriguing that hGR/HPR should be identified as an interacting partner of hSVCT1 given that both proteins play a role in regulating cellular oxalate levels. Oxalate is a degradation product of vitamin C, such that increased AA uptake would likely increase oxalate levels. Some reports find that megadoses of vitamin C yield hyperoxaluria (25), although this finding is controversial (14). Conversely, hGR/HPR serves to limit cellular oxalate production by decreasing endogenous glyoxylate. The physical association of these proteins with coupled activities and opposing outcomes on cellular oxalate levels may therefore represent an adaption to minimize the risk of hyperoxalosis in scenarios of enhanced vitamin C absorption. Hence, the physical coupling of hSVCT1 with hGR/HPR localizes independent factors that are involved in cellular oxalate production and metabolism within the same protein complex and establishes hGR/HPR as a novel associated protein of hSVCT1.

The presence of hSVCT1 and hGR/HPR on a population of intracellular structures may add further significance both for the mechanism of organellar accumulation of ascorbate as well as for the local regulation of glyoxylate/glycolate concentrations (Fig. 5). For example, glyoxylate supply to peroxisomes is important for its metabolism (2, 18), and dysfunction in peroxisomal alanine:glyoxylate aminotransferase is known to yield primary hyperoxaluria type 1 (10). Similarly, ascorbate plays an important role in peroxisome biology as both an antioxidant and an enzymatic cofactor. About a third of all antioxidants and an enzymatic cofactor. About a third of all hydrogen peroxide generated in hepatocytes derives from peroxisomal oxidases (6), and ascorbate is used to scavenge exogenous radicals in the peroxisome matrix. Ascorbate also acts as a cofactor in α-oxidation reactions catalyzed by phytanoyl-CoA hydroxylase (9), an enzyme associated with the metabolic disorder Refsum disease (17). However, little is known about the mechanism of AA accumulation into different organelles although carrier-mediated systems have been described in mitochondria and endoplasmic reticulum (reviewed in Refs. 22 and 40). The demonstration of association between hSCVT1 and hGR/HPR on organellar membranes provides precedence for a possible route for organellar AA accumulation and for local glyoxylate/glycolate flux control. However, additional studies are needed to define the precise organelar population where hSVCT1 and hGR/HPR interact and whether this association is regulatable by physiological factors.

In summary, our data report for the first time the identification of hGR/HPR as an interacting protein partner of hSVCT1.
in human liver cells, which appear to play a role in hSVCT1 transport physiology and cell biology.

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DISCLOSURES
No conflicts of interest or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
6. Boveris A, Oshino N, Chance B. Oxidation of pyridine nucleotide by 10.220.33.5 on July 1, 2017 http://ajpgi.physiology.org/ Downloaded from


