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

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Glyoxalate reductase/hydroxypyruvate reductase interacts with the sodium-dependent vitamin C transporter-1 to regulate cellular vitamin C homeostasis. Am J Physiol Lung Cell Mol Physiol 304: G1079–G1086, 2013. First published April 18, 2013; doi:10.1152/ajplung.00090.2013.—The human sodium-dependent vitamin C transporter (hSVCT1) contributes to cellular uptake of ascorbic acid (AA). Although different aspects of hSVCT1 cell biology have been extensively studied, nothing is currently known about the broader hSVCT1 interactome that modulates its role in cellular physiology. Here, we identify the enzyme human glyoxalate reductase/hydroxypyruvate reductase (hGR/HPR) as an hSVCT1-associated protein by yeast two-hybrid (Y2H) screening of a human liver cDNA library. The interaction between hSVCT1 and hGR/HPR was further confirmed by in vitro GST pull-down assay, in vivo coimmunoprecipitation and mammalian two-hybrid firefly luciferase assays. This interaction had functional significance as coexpression of hGR/HPR with hSVCT1 led to an increase in AA uptake. Reciprocally, siRNA-mediated knockdown of endogenous hGR/HPR led to an inhibition of AA uptake. Given that oxalate is a degradation product of vitamin C and hGR/HPR acts to limit cellular oxalate levels, this association physically couples two independent regulators of cellular oxalate production. Furthermore, confocal imaging of human liver HepG2 cells coexpressing GFP-hSVCT1 and hGR/HPR-mCherry demonstrated that these two proteins colocalize within a subpopulation of intracellular organelles. This provides a possible molecular basis for organelar AA transport and regulation of local glyoxalate/glycolate concentration in the vicinity of organelle membranes.

vitamin C; uptake; transport; yeast two-hybrid

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, nonalcoholic 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 SLCD31A1 and SLCD31A2 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/hydroxypyruvate 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 glyoxylate to glycolate and hydroxypruvrate to r-glycerate. The reduction of glyoxylate to glycolate 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). Geneticin (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
Table 1. Combination of primers used to prepare the constructs by PCR and real-time PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward and Reverse Primers (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian two-hybrid assay</td>
<td>hSVCT1</td>
</tr>
<tr>
<td>hSVCT1</td>
<td>GCTGGACGTTATAGGCGCCGCAGAGGAC</td>
</tr>
<tr>
<td>hGR/HPR</td>
<td>GCCAACTGTATAGGGCGCCGACCAGGAC</td>
</tr>
<tr>
<td>Coimmunoprecipitation assay</td>
<td>hSVCT1</td>
</tr>
<tr>
<td>hSVCT1</td>
<td>CCCAACGTATAGGGCGCCGACCAGGAC</td>
</tr>
<tr>
<td>hGR/HPR</td>
<td>GCCAACTGTATAGGGCGCCGACCAGGAC</td>
</tr>
<tr>
<td>Confocal studies</td>
<td>hSVCT1</td>
</tr>
<tr>
<td>hSVCT1</td>
<td>CCCAACGTATAGGGCGCCGACCAGGAC</td>
</tr>
<tr>
<td>hGR/HPR</td>
<td>CCCAACGTATAGGGCGCCGACCAGGAC</td>
</tr>
<tr>
<td>Real-time PCR studies</td>
<td>hSVCT1</td>
</tr>
<tr>
<td>hSVCT1</td>
<td>TGCATCTGCTGTTCTCCGCACTACTCTT</td>
</tr>
<tr>
<td>hSVCT2</td>
<td>TCTTTGCTGCTTGTGATTGTTGAGT</td>
</tr>
<tr>
<td>hGR/HPR</td>
<td>GACTGCGGAGGCCGCGGAGGAC</td>
</tr>
<tr>
<td>hβ-actin</td>
<td>AGCTGCGGAGGCCGCGGAGGAC</td>
</tr>
</tbody>
</table>

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 (underlined text), and KpnI (italics) were added to the hSVCT1 and hGR/HPR primers to allow subsequent subcloning into the GFP-C1, pFLAG, mCherry, pBIND, and pACT vectors.
GAUAAtt, antisense: UAUCAGAAUACUCUUCUUCC(C)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, hSVCT2, 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, hSVCT2, 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-amino triazole (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 immunoprecipititates 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.

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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).

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
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-
disorder Refsum disease (17). However, little is known about CoA hydroxylase (9), an enzyme associated with the metabolic metabolism of phytanoyl-CoA. Additional experiments are needed to define the precise organellar population that hSVCT1 and hGR/HPR interact on. hSVCT1 (gray) transports vitamin C across the cellular membrane, as hGR/HPR (black box) catalyzes conversion of ascorbic acid to dehydroascorbic acid. The presence of hSVCT1 and hGR/HPR on a population of intracellular structures may add further significance both for 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 organelar 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.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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