Mammalian Selenocysteine Lyase Is Involved in Selenoprotein Biosynthesis

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Summary Selenocysteine lyase (SCL) catalyzes the decomposition of l-selenocysteine to yield l-alanine and selenium by acting exclusively on l-selenocysteine. The X-ray structural analysis of rat SCL has demonstrated how SCL discriminates l-selenocysteine from l-cysteine on the molecular basis. SCL has been proposed to function in the recycling of the micronutrient selenium from degraded selenoproteins containing selenocysteine residues, but the role of SCL in selenium metabolism in vivo remains unclear. We here demonstrate that the 72Se-labeling efficiency of selenoproteins with 72Se-labeled selenoprotein P (Sepp1) as a selenium source was decreased in HeLa cells transfected with SCL siRNA as compared to the cells transfected with control siRNA. Immunocytochemical analyses showed high SCL expression in kidney and liver cells, where selenocysteine is recovered from selenoproteins. Mature testes of mice exhibited a specific staining pattern of SCL in spermatids that actively produce selenoproteins. However, SCL was weakly expressed in Sertoli cells, which receive Sepp1 and supply selenium to germ cells. These demonstrate that SCL occurs in the cells requiring selenoproteins, probably to recycle selenium derived from selenoproteins such as Sepp1.

Key Words selenocysteine lyase, selenium, selenoprotein synthesis, RNAi, immunohistochemistry

L-Selenocysteine (1), the 21st amino acid, is cotranslationally incorporated into selenoproteins at UGA codons (2). Decoding of the UGA as selenocysteine rather than as a termination codon requires a specific stem-loop structure in selenoprotein mRNAs, known as a selenocysteine insertion sequence (SECIS) element (1), several trans-acting factors, and a unique tRNA with an anticodon complementary to UGA. A specific SECIS-binding protein, SBP2, is required for the insertion of selenocysteine into a selenoprotein (3). Selenocysteine is derived from serine through enzymatic replacement on selenocysteine tRNA of its β-hydroxyl group by selenol (4). Monoselenophosphate, which is produced by selenophosphate synthetase 2 (SPS2) from selenide and ATP, is the active donor of selenium and is an essential precursor for the selenol of selenocysteyl-tRNA (5). Thus, selenide production is an important step in selenoprotein biosynthesis in mammals.

The selenocysteine residues in selenoproteins are important selenium sources in mammals (6). Selenoprotein P (Sepp1) is unique in that its mRNAs in different mammalian species encode 10–12 selenocysteine residues (7). Sepp1 carries up to 60% of the selenium in plasma, and a previous study has suggested that it is the supplier of selenium to the brain, testis, and kidney (8). Especially in testis, it is important for spermatids to receive selenium in the form of selenocysteine (9).

During studies of the enzymatic synthesis of l-selenocysteine in rat liver (10), we discovered selenocysteine lyase (SCL) as the first enzyme that specifically acts on selenium compounds: SCL does not decompose l-cysteine (11). We recently showed that rat SCL acts as a sorter for selenium from the mixture of selenium and sulfur by means of Cys375 on the flexible loop to direct l-selenocysteine but not l-cysteine to the correct position at the active site of SCL (12). The structural basis for discrimination between selenocysteine and cysteine by the enzyme is clear, but the physiological role of SCL remains unclear. Therefore, we examined in this study the effect of SCL knockdown in HeLa cells on the selenoprotein synthesis. Further, we demonstrated that SCL is expressed in a cell-specific manner in the seminiferous tubules, dominantly in Leydig cells and spermatids. The results of the present study suggest that SCL serves to provide selenium from selenoproteins degraded to those newly synthesized through specific decomposition of l-selenocysteine.

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EXPERIMENTAL PROCEDURES

Materials. Rat SCL was expressed in Escherichia coli BL21(DE3) and purified to homogeneity for antigen, which was used to produce Rabbit anti-SCL antibody. Immune serum was affinity-purified in antigen-agarose affinity columns. Mouse monoclonal anti-GAPDH antibody was purchased from Ambion (Austin, TX). Rabbit anti-GPX1 antibody was obtained from Cell Signaling Technology (Danvers, MA). L-Selenocystine was purchased from Sigma (St. Louis, MO). Restriction and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). [75Se]Selenite was obtained from the University of Missouri Research Reactor Facility (Columbia, MO). All the other reagents used were of analytical grade.

Constructs. The full-length mouse SCL cDNA was PCR-amplified from C57/BL6 cDNA using the primers 5'-ATAAAAATGCTGACCATGTGTGCTGTCGGCTCTCAGGCGGCACAG-3' (forward) and 5'-GGTACCGATTTGCCAGCTTATTTCTAGTGGAAACCCATCCTTCAT-3' (reverse). The PCR product was digested with Nhel/KpnI and ligated into the Nhel/KpnI sites of pcDNA3.1/Zeo(+) (Invitrogen Corporation, Carlsbad, CA) to yield pcDNA-SCL, an SCL-overproducing vector. For the green fluorescent protein (GFP) fusion construct, the GFP gene was amplified from pEGFP (Clontech) using the primers 5'-CACGACAGCATATGGTGTTTCTAAGGGAGAAGA-3' (forward, Ndel site) and 5'-CACGACAGCTATGGTTAAGAGAGAAGA-3' (reverse, EcoRI site). For the SCLGFP protein, mouse SCL cDNA fragment was PCR-amplified from pcDNA-SCL using the primers 5'-CACGACAGCATATGGTGTTTCTAAGGGAGAAGA-3' (forward, Ndel site) and 5'-CACGACAGCTATGGTTAAGAGAGAAGA-3' (reverse, Ndel site). The PCR products were cloned into the Nhel/EcoRI sites of pcDNA3.1/Zeo(–). Constructs were verified by dideoxy sequencing to ensure that no mutations were introduced by PCR.

Cell culture conditions and transient transfections. Transient transfections in HeLa cells were carried out using Lipofectamine LTX (Invitrogen Corporation). Three days prior to transfection, the cells were plated on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 30-mm culture dishes. Cells were transfected with the expression plasmids according to the manufacturer's instructions.

RNAi. Human SCL siRNA with a sense-sequen 5'-AATCGTGGGCGACAAGTTTTA-3' was purchased from Qiagen (Tokyo, Japan). Alexa Fluor 488-labeled non-silencing duplex siRNA (Qiagen) with a sense sequence of 5'-UUUUCUCGGAACGUUGCAGUdTdT-3' was used as the control. HeLa cells were transfected with 5 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Corporation) according to the manufacturer’s instructions. To study the effects of selenium supplementation, graded amounts of sodium selenite, L-selenocysteine, and L-selenomethionine were added to the culture media 2 d after transfection. The cells were harvested at various time points after transfection, solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. Samples were diluted in an SDS sample buffer containing 50 mM dithiothreitol and heated at 95˚C for 5 min. SDS-PAGE was performed on 4–20% acrylamide gels (Wako Pure Chemical Industries, Ltd., Kanagawa, Japan). Polypeptides were visualized with Coomassie Blue or transferred to polyvinylidene fluoride membranes for Western blot analyses. The blots were rinsed in phosphate-buffered saline (PBS) and blocked with 5% skim milk in PBS. They were then incubated with anti-SCL antibody diluted 1:6,000 in Tris buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.5) containing 0.1% Tween-20 (TBST). The blots were washed 4 times with TBST and then incubated with secondary antibody (horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG; Bio-rad) diluted 1:100,000 in TBST. Then, the blots were washed in TBST, developed in ECL-plus reagent (GE Healthcare, Upppsala, Sweden), and exposed to X-ray films.

Preparation of 75Se-labeled Sepp1. 75Se labeling of Sepp1 was performed essentially according to Tujebajewa et al. (13) with some modifications. Briefly, cells of the human liver carcinoma cell line HepG2 were maintained in DMEM supplemented with 10% FBS and 300 mM sodium selenite for 3 d prior to changing the medium to serum-free DMEM medium. Cells were rinsed with FBS-free DMEM and then incubated in FBS-free DMEM with [75Se]selenite (39 μCi/ml) for 24 h. The medium, containing 75Se-labeled secreted Sepp1, was collected.

Taking advantage of the histidine-rich nature of Sepp1, nickel-agarose affinity chromatography has been utilized for purification of the protein (13). Nickel-agarose chromatography was performed using a nickel-nitrilotriacetic acid spin column (Qiagen). The medium from the 75Se-labeled cells (up to 3 column volumes) were allowed to percolate into a nickel-nitrilotriacetic acid spin column equilibrated with protein-binding (PB) buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 10 mM mercaptoethanol, and 10% glycerol. The nickel-nitrilotriacetic acid spin column resin was washed at least 3 times with PB buffer containing 0.5 M NaCl. 75Se-labeled Sepp1 was eluted from the columns with PB buffer containing 0.5 M NaCl and 100–400 mM imidazole.

Immunocytochemistry. Mouse liver and kidney were fixed with 4% formaldehyde in 0.1 M sodium phosphate (pH 7.4) for 1 h at 4˚C, rinsed in phosphate buffer, and infiltrated overnight at 4˚C in phosphate buffer containing 20% sucrose. The tissues were then immersed in OCT (optimal cutting temperature compound; Fisher Scientific, Atlanta, GA), frozen in liquid nitrogen, and then stored at −70˚C until sectioning. The cryosections were rinsed with TBST2 (20 mM Tris-HCl, pH 8.0; 150 mM sodium chloride; 0.05% Tween-20, and 0.025% sodium azide) and blocked with TBST2 con-
taining 1% BSA and 0.1% glycine for 1 h. The sections were then incubated with each antibody in blocking solution for 1 h at room temperature. Control sections were incubated with equivalent levels of nonimmune rabbit IgG. The sections were then washed in TBST 3 times for 5 min each and incubated for 1 h in affinity-purified Cy3-goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in the blocking solution. Hoechst-33258, used to stain DNA, and Alexa 488-phalloidin, used to stain F-actin (Invitrogen Corporation), were added to the secondary antibody solution. The slides were washed 3 times for 5 min each with TBST2 and mounted in 50% glycerol in TBS (150 mM NaCl and 10 mM Tris-HCl, pH 7.5). The specimens were examined by phase contrast and epifluorescence microscopy, and digital images of the experimental and control samples were obtained using identical photographic settings.

Mouse testis was fixed at 4˚C with 4% formaldehyde in 0.1 M sodium phosphate (pH 7.4). The tissue was rinsed in phosphate buffer containing 70% ethanol and paraffinized. Deparaffinized sections were incubated with a primary antibody and with an Alexa Fluor-conjugated secondary antibody using protocols similar to those described above.

**RESULTS**

**SCL siRNA targets SCL mRNA**

Western blot analysis was used to determine the effect of SCL siRNA on the steady-state levels of the SCL gene expression in HeLa cells. SCL siRNA effectively knocked down the expression of SCL mRNA in the HeLa cells 3 d after transfection (Fig. 1A), and the knockdown effect of SCL siRNA was observed for at least 5 d (Fig. 1B). The SCL-depleted cells did not exhibit any changes in morphology.

**Effect of SCL depletion on selenoprotein expression**

To assess the effect of targeted removal of SCL on sele-
noprotein expression. HeLa cells transfected with SCL siRNA or control siRNA were grown in a medium containing 10% FBS. The SCL expression was decreased to 20% by the SCL siRNA transfection as compared with the level of the control. The GPx1 expression examined by Western blotting decreased to 24% at 5 d after SCL siRNA transfection as compared with the corresponding control (Fig. 2A). Next, to assess the effect of specific selenium sources on selenoprotein expression in the SCL-knockdown cells, HeLa cells were cultured in a medium supplemented with selenite, selenocystine (serves as selenocysteine by reduction), or selenomethionine (300 mM each). In HeLa cells, the SCL expression level was the same either in the presence or absence of the above selenium compounds (Fig. 2B). GPx1 was poorly expressed in HeLa cells in the absence of supplemented selenium, but its expression was slightly induced in the media supplemented with the selenium compounds (Fig. 2B). In the selenocystine-supplemented medium, the expression level of GPx1 in the SCL siRNA-transfected cells decreased to 46% of that in the cells transfected with control siRNA. Thus, SCL participates in the incorporation of selenocysteine into GPx1. In contrast, GPx1 was fully expressed irrespective of SCL siRNA treatments in the cells cultivated in selenite- and selenomethionine-supplemented media (expression levels: 105% and 92%, respectively, of that in the control siRNA-transfected cells).

**Effect of SCL depletion on selenoprotein production during 75Se-labeled Sepp1 supplementation**

We used radiolabeled selenium to assess selenoprotein production in the cells lacking SCL. SCL depletion did not affect the selenoprotein labeling efficiency using selenite as a selenium source (data not shown). This observation is consistent with the result that SCL participated in the incorporation of selenocysteine, but not selenite, into Gpx1 (Fig. 2B). Then, HeLa cells transfected with the SCL siRNA or control siRNA were cultured in the presence of 75Se-labeled Sepp1 for 24 h. Homogenates of SCL-depleted and control cells cultured with 75Se-labeled Sepp1 were subjected to reducing SDS-PAGE (Fig. 3). Coomassie Blue staining demonstrated equivalent loading of proteins of HeLa cell homogenates (Fig. 3, left panel). Autoradiograms of 75Se-labeled HeLa cell homogenates revealed a major radiolabeled band that migrated at 60 kDa, which corresponds to the size of thioredoxin reductase, and minor labeled bands that migrated at 25, 20, and 15 kDa (Fig. 3, right panel). SCL siRNA-transfection had a pronounced effect on the 75Se-labeling efficiency of selenoproteins: the labeling efficiency in the SCL siRNA-transfected cells decreased to 63% of the efficiency of the control siRNA-treated cells (Fig. 3, right panel). These indicate that SCL is required for utilization of selenium derived from Sepp1 for the biosynthesis of selenoproteins in HeLa cells.

**Cell-specific SCL expression in liver and kidney**

We previously demonstrated that SCL is abundant in liver and kidney (14). To examine the protein expression of SCL, cryosections of the mouse liver and kidney were immunostained with a polyclonal rabbit anti-SCL antibody. The anti-SCL antibody specifically reacted to liver and kidney SCL (Fig. 4A). Immunofluorescence staining of the liver with anti-SCL antibody revealed nuclear staining in the hepatocytes (Fig. 4B). The staining was observed throughout the liver lobule. In the kidney, the nuclei of the renal proximal tubule cells revealed intense staining, but less intense staining was detected in the distal tubules, and the glomerulus cells showed no detectable staining (Fig. 4D). A nonimmune IgG showed no signal (Fig. 4C and 4E).

**SCL is expressed in Leydig cells and spermatids**

Selenoprotein can be a source of L-selenocysteine by proteolysis, and we examined seminiferous tubules as a model for the visualization of the steps involved in selenoprotein production. Sepp1 is a selenium source for spermatids, and ApoER2, a receptor expressed on Sertoli cells, is required for its uptake (15). To identify the cell types of the seminiferous tubules, staining was carried out with anti-SCL antibody (Fig. 5). The nuclei were stained using 4',6-diamidino-2-phenylindole. Merged immunofluorescence staining of the testis with anti-SCL antibody revealed staining in the nuclei of Leydig cells and spermatids and to a lesser extent in the regions surrounding the Sertoli cells. In particular, the signal was observed when the spermatids were in the meiotic prophase, and it disappeared at step 10 of spermiogenesis.

To investigate the cellular distribution of SCL in living cells, both N- and C-terminal GFP fusion constructs
were generated. SCLGFP was predominantly present in the nucleus of HeLa cells 48 h after transfection, whereas GFP expression alone resulted in homogenous distribution throughout nucleus and cytosol (Fig. 5B). Because N- or C-terminal GFP fusion can affect localization, a GFPSCL construct was also used to confirm the subcellular localization of SCL in the nucleus. We found that GFPSCL fusion protein was also present in the nucleus (data not shown). When mouse hepatoma (Hepa 1–6) and human embryonic kidney (HEK) cells were examined, the same results were obtained (data not shown). These indicate that SCL is localized in the nucleus, even though it does not have obvious nuclear localizing signals.

DISCUSSION

This is the first study to demonstrate that SCL mediates selenoprotein biosynthesis when cell culture is carried out in the presence of selenium in the form of L-selenocysteine. Interestingly, the reduced expression of GPx1 caused by SCL depletion in the presence of selenocysteine as a selenium source was restored when the culture was supplemented with selenite or selenomethionine (Fig. 2B). This observation suggests that transformation of selenomethionine to selenocysteine via the transsulfuration pathway is not the only mechanism by which selenium is released from selenomethionine (Fig. 6A). Selenomethionine might be decomposed by cystathionine γ-lyase (CGL) to generate methane selenol and used as a selenium source for selenoproteins as...
reported previously (16, 17). Targeted removal of SCL resulted in a significant decrease of the $^{75}$Se-labeling efficiency of selenoproteins, indicating that SCL is required for the utilization of selenocysteine derived from Sepp1 in HeLa cells. Immunohistochemical studies with an anti-SCL antibody demonstrated the presence of SCL in the mouse liver, kidney, and testis, in which tissues selenium plays an important role. Further, nuclei of hepatocytes showed the presence of SCL. While nuclei of renal proximal tubules showed the presence of SCL, SCL was absent in the renal glomerulus. In the kidney, Sepp1 is taken up by the proximal tubules, which secrete GPx3 (18). Deletion of Sepp1 decreased the renal selenium content, indicating that some of selenocysteine in Sepp1 is utilized for the synthesis of GPx3 (8). Immunostaining of the mouse testis demonstrated predominant occurrence of SCL in pachytene spermatocytes and Leydig cells while Sertoli cells displayed lower levels of SCL.

Recent studies using X-ray fluorescence microscopy revealed that Sepp1 is a major selenium source in germ cells, and that selenium is highly enriched in germ cells (19). Sepp1 bound to ApoER2 is internalized by Sertoli cells, and its selenocysteine is probably decomposed by
SCL in germ cells and utilized for selenoprotein synthesis. Sepp1-knockout mice showed infertility, which cannot be recovered by supplementation with selenite (9). The CGL expression levels in testis were found to be low, and selenomethionine is not probably used as a selenium source in germ cells (20). The testis has tight junctions, i.e., the blood-testis barrier, and this selenium transfer system involving Sepp1, similarly to the transfer of iron mediated by testicular transferrin (21), is probably essential for supply of selenium to germ cells.

SPS2 converts selenide to selenophosphate (5), and SBP2 is required for selenoprotein biosynthesis in germ cells (Fig. 6B). Selenophosphate synthetase 1 (SPS1) was originally considered to play a role in selenium metabolism in mammals (22), but an in vitro study recently showed that SPS1 is unable to synthesize selenophosphate (5). The nuclear localization of mammalian SCL was an unexpected finding. Selenocysteine is a very rare amino acid in cells. Thus, its selenium is likely recycled: free selenium is released from selenoprotein metabolites (5). We have observed that SCL binds to the selenium of decomposed selenocysteine at the active site by forming perselenide at a cysteine residue (12). Therefore, the selenium atom bound to SCL can migrate with SCL itself into the nucleus and provide selenium to the selenoprotein biosynthesis machinery complex therein. Further studies are required to show the mechanism for the selection of the selenium-bound form of SCL from free SCL.

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REFERENCES


