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Long Isoform Mouse Selenoprotein P (Sepp1) Supplies Rat Myoblast L8 Cells with Selenium via Endocytosis Mediated by Heparin Binding Properties and Apolipoprotein E Receptor-2 (ApoER2)*

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Background: ApoER2 endocytosis of Sepp1 supplies testis and brain with selenium, but the mechanism of supply to other tissues is not known.
Results: Sepp1 supplies selenium to heart and skeletal muscle cell lines via apoER2 and many tissues express apoER2.
Conclusion: ApoER2 endocytosis of Sepp1 supplies selenium to many tissues.
Significance: ApoER2 uptake of Sepp1 likely regulates selenium distribution in the whole body.

In vivo **studies have shown that selenium is supplied to testis and brain by apoER2-mediated endocytosis of Sepp1. Although cultured cell lines have been shown to utilize selenium from Sepp1 added to the medium, the mechanism of uptake and utilization has not been characterized. Rat L8 myoblast cells were studied. They took up mouse Sepp1 from the medium and used its selenium to increase their glutathione peroxidase (Gpx) activity. L8 cells did not utilize selenium from Gpx3, the other plasma selenoprotein. Neither did they utilize it from Sepp1$^{\Delta 240-361}$, the isoform of Sepp1 that lacks the selenium-rich C-terminal domain. To identify Sepp1 receptors, a solubilized membrane fraction was passed over a Sepp1 column. The receptors apoER2 and Lrp1 were identified in the eluate by mass spectrometry. siRNA experiments showed that knockdown of apoER2, but not of Lrp1, inhibited ^{75}Se uptake from ^{75}Se-labeled Sepp1. The addition of protamine to the medium or treatment of the cells with chlorate also inhibited ^{75}Se uptake. Blockage of lysosome acidification did not inhibit uptake of Sepp1 but did prevent its digestion and thereby utilization of its selenium. These results indicate that L8 cells take up Sepp1 by an apoER2-mediated mechanism requiring binding to heparin sulfate proteoglycans. The presence of at least part of the selenium-rich C-terminal domain of Sepp1 is required for uptake. RT-PCR showed that mouse tissues express apoER2 in varying amounts. It is postulated that apoER2-mediated uptake of long isoform Sepp1 is responsible for selenium distribution to tissues throughout the body.**

Animal cells must acquire selenium for synthesis of their selenoproteins. Some tissues tolerate selenium deficiency reasonably well, but others, *e.g.* testis and brain, require a reliable supply of the element to support their function and viability. In recent years Sepp1[2] has been shown to supply selenium to those high-need tissues (1, 2).

Sepp1 is a complex protein with two major domains (3, 4). The N-terminal domain, two-thirds of the primary structure, contains one selenocysteine residue in a redox motif and several potential heparin-binding sites. The shorter C-terminal domain contains nine selenocysteine residues. Four Sepp1 isoforms that share the same N-terminal amino acid sequence but have different termination sites have been purified from rat plasma (5). The shortest isoform (Sepp1$^{\Delta 240-361}$) is the N-terminal domain that terminates at the second selenocysteine. The longest isoform is the full-length protein, and the other isoforms terminate at the third and seventh selenocysteine residues. The individual isoforms have not been quantified, and the reasons for their existence are not known. There is evidence that isoforms also exist in mouse and human plasma (6, 7), but they have not been characterized or quantified.

Sepp1 and Gpx3 account for greater than 97% of mouse plasma selenium, but of the two, only Sepp1 appears to have a role in selenium transport (8). Mice with deletion of Sepp1 or of both these selenoproteins survive only when fed a high selenium diet (1), indicating that small molecule forms of selenium are able to supply the element to high-need tissues when selenium supply is abundant. Sepp1, however, is needed to ensure appropriate tissue selenium distribution when selenium supply is normal or deficient.

Receptor-mediated endocytosis of Sepp1 supplies the element to tissues that depend on it for their function and survival. Spermatogenesis, a selenium-dependent process, is supported by apoER2-mediated endocytosis of Sepp1 by Sertoli cells (9). ApoER2, a member of the low density lipoprotein receptor fam-

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[2] The abbreviations used are: Sepp1, selenoprotein P; apoER2, apolipoprotein E receptor-2; Gpx, glutathione peroxidase; HSPG, heparin sulfate proteoglycan; Lrp1, low density lipoprotein receptor-related protein; ANOVA, analysis of variance; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

TABLE 1
Primer sequences used for RT-PCR quantitation
Hprt, hypoxanthine phosphoribosyltransferase.

Genes	PCR primer sequences (5′–3′)	GenBank™ accession number
Rat cell lines		
apoER2	GCGTTTGTACTGGGTGGACT GAAAATGGCCTCATTCTCCA	NM_009155
Lrp1	GTGAGGGTGTGGGTTCTTTTCTC ATTCCAACAGCCAGTGACGT	NM_001130490
cyclophilin A	CCCACCGTGTTCTTCGACAT CCTTGTCTGCAAACAGCTCAAA	NM_017101
Mouse tissues		
apoER2	AGATGGGCTCAACAGTCACC AGTGGGCGATCATAGTTGCT	NM_001080926
Hprt	TCCTCCTCAGACCGCTTTT CCTGGTTCATCGCTAATC	NM_013556

ily, also maintains brain selenium (10). Megalin, another low density lipoprotein receptor family member, facilitates endocytosis of filtered forms of Sepp1 by kidney proximal convoluted tubule cells (11). It has not been reported whether Sepp1 supplies other tissues with selenium through a receptor-mediated mechanism.

Although cultured Jurkat cells have been shown to take up selenium supplied as Sepp1, the mechanism of that acquisition was not presented (12). The work reported here characterizes the uptake and utilization of mouse Sepp1 selenium by the rat myoblast L8 cell line. Unexpectedly, L8 cells were found to take up Sepp1 by apoER2-mediated endocytosis, implying that apoER2 has a major role in systemic selenium physiology.

MATERIALS AND METHODS

Animals—These studies used sera from mice with deleted or altered serum selenoproteins to characterize Sepp1 uptake. The use of selenoproteins synthesized in mice was necessary because synthesis of these selenoproteins in bacterial systems has not yet been achieved.

Mice used in these experiments were taken from our C57BL/6-congenic *Sepp1*$^{+/-}$ (1), *Sepp1*$^{\Delta 240-361/+}$ (6), and *Gpx3*$^{-/-}$ (8) colonies. They were housed in our Association for Assessment and Accreditation of Laboratory Animal Care International-approved facility that has a 12-h/12-h light/dark cycle. Food and tap water were provided *ad libitum*. The diet fed was *Torula* yeast-based and was formulated to our specifications (13) by Harlan Teklad (Madison, WI). It was used in the basal, selenium-deficient form and in a selenium-adequate form supplemented with 0.25 mg (1.0 mg for mice with *Sepp1* deleted) selenium per kg as sodium selenite. Care and use of animals in these experiments conformed to National Institutes of Health guidelines for animal care and use in research. The Vanderbilt Institutional Animal Care and Use Committee approved all animal protocols.

Materials—Rat myoblast L8 and cardiomyocyte H9c2 cell lines were purchased from the American Type Culture Collection (Manassas, VA). The rat monoclonal antibody 9S4 against the N-terminal domain of mouse Sepp1 was previously described (6). The hybridoma that produces 9S4 was a gift from Dr. T. Naruse of Kaketsuken, The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). ^{75}Se-labeled selenite, specific activity ~2100 μCi/μg of selenium, was purchased from the University of Missouri Research Reactor Facility (Columbia, MO).

The primers, target siRNAs, and Universal Negative Control#1 siRNA (Table 1) were purchased from Sigma. High Capacity cDNA Reverse Transcriptase kit and 2× Power SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Lipofectamine RNAiMAX, NuPAGE gels, TRIzol reagent, and NuPAGE sample buffer were purchased from Invitrogen. RT-PCR reagents were purchased from Applied Biosystems.

Bafilomycin A1 was purchased from Tocris Bioscience (Bristol, UK); chloroquine diphosphate and chlorpromazine were purchased from MP Biomedicals (Solon, OH); protamine was purchased from Sigma; sodium chlorate and nystatin were purchased from Fisher. Other chemicals were reagent grade or better.

Selenium and Gpx Assays—Selenium was determined in tissues and serum by the method of Koh and Benson (14) as modified by Sheehan and Gao (15). Gpx activity was measured in cell lysate as previously described using 0.25 mM H_2O_2 as substrate (1). Protein concentrations were determined using bicinchoninic acid (BCA) assay reagents (Pierce).

^{75}Se Labeling of Serum Selenoproteins—Mice were switched from a selenium-adequate diet to a selenium-deficient diet 2 days before intraperitoneal injection of 20 μCi of ^{75}Se-labeled selenite. Four hours after injection, mice were anesthetized with isoflurane, and blood was collected from the vena cava and allowed to clot. Serum was separated by centrifugation and dialyzed overnight against PBS at 4 °C before being added to cell culture medium. ^{75}Se was quantified using a γ-counter (PerkinElmer Life Sciences model 1480 Wizard 3" gamma counter, Shelton, CT).

Cell Culture—L8 and H9c2 cells were maintained in DMEM/F-12 medium containing 10% FBS at 37 °C under 95% air, 5% CO_2. All experiments were carried out in medium that did not contain FBS.

For inhibition of heparan sulfate synthesis, cells were preincubated for 24 h in serum-free medium at several concentrations of sodium chlorate. For inhibition of lysosome acidification, cells were incubated in serum-free medium containing 100 nM bafilomycin A1 or 100 μM chloroquine beginning 30 min before the start of the experiments (16, 17).

^{75}Se Uptake Determination—L8 or H9c2 cells were trypsinized and collected by centrifugation. They were resuspended in serum-free DMEM/F-12 medium at 3.5×10^5 cells/well in a volume of 500 μl/well and cultured for 48 h before use in the

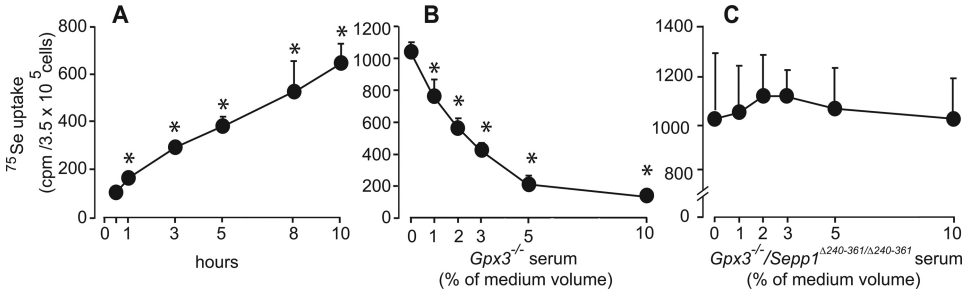

FIGURE 1. **Uptake of ^{75}Se from ^{75}Se-labeled Sepp1 by L8 cells and inhibition of that uptake by serum selenoproteins.** L8 cells were incubated in medium containing 2% *Gpx3*$^{-/-}$ mouse serum that contained ^{75}Se-labeled Sepp1 as the only selenoprotein. *A*, the ^{75}Se content of the cells was determined at intervals of up to 10 h of incubation. *B*, the effect of non-labeled Sepp1 on uptake of ^{75}Se from ^{75}Se-labeled Sepp1 was determined. ^{75}Se uptake was determined in cells cultured for 5 h with *Gpx3*$^{-/-}$ mouse serum added to the medium in different concentrations. *C*, the effect of non-labeled Gpx3 and the shortest isoform of Sepp1 (Sepp1$^{\Delta 240-361}$) on uptake of ^{75}Se from ^{75}Se-labeled Sepp1 was determined. ^{75}Se uptake was determined in cells cultured for 5 h with *Sepp1*$^{\Delta 240-361/\Delta 240-361}$ mouse serum added to the medium in different concentrations. All values are the means ($n = 4$) with 1 S.D. indicated by the *brackets*. *Asterisks* indicate values significantly different ($p < 0.05$) from the 30-min value (*panel A*) or the value with no unlabeled serum added (*panels B* and *C*) by Tukey's multiple comparison test applied after 1-way ANOVA of the groups.

^{75}Se uptake assay. The cells were rinsed with PBS 3 times and incubated with 200 μl of DMEM/F-12 containing the ^{75}Se source (^{75}Se-labeled selenite or specific ^{75}Se-labeled serum) for 5 h under humidified 95% air, 5% CO_2 at 37 °C. After incubation, the supernatant was removed, and the cells were washed 3 times with PBS and then lysed with 2% SDS in 25 mM Tris base, 192 mM glycine. ^{75}Se was determined in the lysate. All experiments were performed in duplicate.

Selenium Utilization Determination—Gpx activity was determined in cells as a measure of the utilization of selenium taken up by them. Cells (3.5×10^6/dish) were cultured in 100-mm Petri dishes in 5 ml of serum-free medium for 48 h. Then 5 ml of fresh medium containing a source of selenium was added, and the cells were cultured for an additional 24 h. Cells were rinsed 3 times with PBS, removed by cell scraper in 0.5 ml of lysis buffer (0.1 M phosphate buffer, pH 7.0, containing 2 mM EDTA and 2 mM sodium azide), lysed by sonication, and centrifuged at 12,000 *g* for 15 min at 4 °C. Gpx activity and total protein concentration of the supernatant were determined.

To ensure that equivalent amounts of selenium were added in the form of different selenoproteins, selenium concentrations were determined in dialyzed sera from *Sepp1*$^{-/-}$, *Gpx3*$^{-/-}$, and *Sepp1*$^{\Delta 240-361/\Delta 240-361}$/*Gpx3*$^{-/-}$ mice. Selenoprotein sources of selenium were Sepp1 in *Gpx3*$^{-/-}$ serum containing 328 ng of selenium/ml (4150 nM selenium), Sepp1$^{\Delta 240-361}$ in *Sepp1*$^{\Delta 240-361/\Delta 240-361}$/*Gpx3*$^{-/-}$ serum containing 114 ng of selenium/ml (1440 nM selenium), and Gpx3 in *Sepp1*$^{-/-}$ serum containing 52 ng of selenium/ml (658 nM selenium).

To add a source of selenium equivalent in concentration to the 83 nM selenium as Sepp1 in medium containing 2% *Gpx3*$^{-/-}$ serum, serum containing only Gpx3 (*Sepp1*$^{-/-}$ serum) was added as 15% of the medium to give 99 nM selenium in the medium, and Sepp1$^{\Delta 240-361}$ (*Sepp1*$^{\Delta 240-361/\Delta 240-361}$/*Gpx3*$^{-/-}$ serum) was added as 7% of the medium to give 101 nM selenium in the medium. Sodium selenite was added to the medium in the concentrations indicated.

RNAi Experiments—Transfections with siRNAs were performed with Lipofectamine RNAiMAX according to the procedure recommended by the manufacturer. Cells were transfected in the presence of Universal Negative Control#1 siRNA

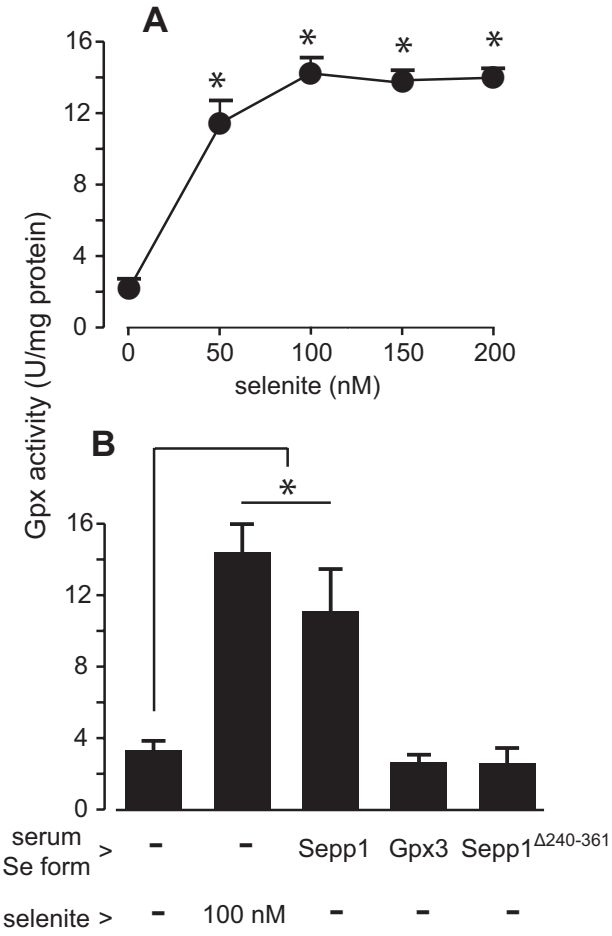

FIGURE 2. **Response of L8 cell Gpx activity to the addition of different selenium sources to the medium.** Selenium-depleted cells were cultured for 24 h with a selenium source and were then assayed for Gpx activity. *A*, selenite at different concentrations was the selenium source. *B*, selenium sources were 100 nM selenite and serum that contained single selenoproteins: Sepp1 (83 nM selenium), Gpx3 (98 nM selenium), and Sepp1$^{\Delta 240-361}$ (101 nM selenium). The values shown are the means of three experiments carried out in duplicate with 1 S.D. indicated by the *bracket*. The *asterisks* in *A* indicate values significantly different ($p < 0.05$) from the value with no selenite added by Tukey's multiple comparison test applied after 1-way ANOVA of the groups and in *B* indicate values that were significantly different ($p < 0.05$) from the basal value by Student's *t* test.

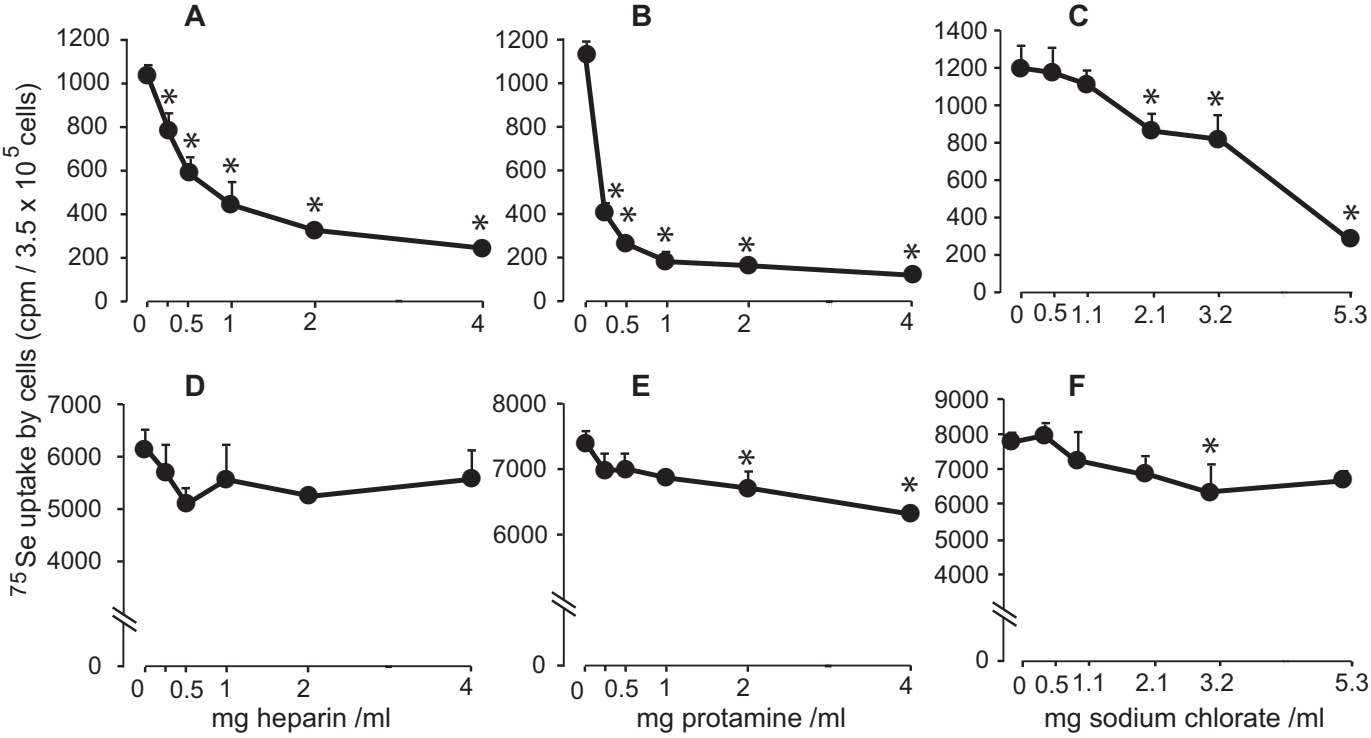

FIGURE 3. **Influence of heparin binding properties on L8 cell ^{75}Se uptake from ^{75}Se-labeled Sepp1.** L8 cells were cultured for 5 h with 2% *Gpx3*$^{-/-}$ mouse serum that contained "all-isoform" ^{75}Se-labeled Sepp1 as the only selenoprotein (*panels A*, *B*, and *C*) or ^{75}Se-labeled selenite at 100 nM (*panels D*, *E*, and *F*). *A* and *D*, heparin was added to the medium at the concentrations indicated. *B* and *E*, protamine was added to the medium at the concentrations indicated. *C* and *F*, chlorate was added to the medium at the concentrations indicated 24 h before the ^{75}Se source was introduced. Values shown are the means ($n = 4$) with 1 S.D. indicated by the *bracket*. *Asterisks* indicate values significantly different ($p < 0.05$) from the initial value by Tukey's multiple comparison test applied after one-way ANOVA of the groups.

or siRNA specific for rat Lrp1 or apoER2 (Table 1). Briefly, a solution containing 1.2 μl of siRNA (20 μM) or 1.2 μl of negative control siRNA (20 μM), 1.5 μl of Lipofectamine RNAiMAX, and 200 μl of serum-free medium was incubated at room temperature for 20 min. One ml of cell suspension (7×10^5 cells/ml in serum-free medium) was added to the siRNA solution, and then it was plated in 4 wells at 300 μl (1.75×10^5 cells) per well. It was incubated in humidified 95% air, 5% CO_2 at 37 °C for 4 h, then 600 μl of serum-free medium was added to each well. Incubation was continued for an additional 44 hours. Silencing was then assessed by measuring levels of Lrp1 and apoER2 mRNA using RT-PCR. Other cells were used in ^{75}Se-labeled Sepp1 uptake experiments.

Affinity Purification and Identification of Sepp1-binding Proteins by Mass Spectrometry—Preparation of a rat Sepp1 column and purification of Sepp1-binding proteins were described previously (9). Briefly, a membrane preparation was prepared from L8 cells. Trypsinized cells were washed with PBS, suspended in ice-cold TNI (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 2 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium fluoride, 1 mM sodium vanadate, and 0.05% sodium azide), and homogenized with a Polytron homogenizer. The suspension was centrifuged at 11,000 *g* for 30 min, the supernatant was discarded, and the pellet was washed by resuspension in TNI followed by repeat centrifugation. Membrane proteins were solubilized at 4 °C in TNI containing 0.2% Triton X-100, and insoluble material was pelleted under the same centrifugation conditions as above. The Triton-soluble fraction was adjusted to 1 mM each $CaCl_2$ and $MgCl_2$ and then incubated with the AminoLink resin with bound rat Sepp1. The resin was packed into a column and washed with 10 volumes of TNI followed by 10 volumes of 1 M NaCl, 1 mM Tris-HCl, pH 7.5. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5. Proteins in the glycine-eluted fraction were trypsin-digested into peptides that were subsequently analyzed using multidimensional high performance liquid chromatography-coupled mass spectrometry (MudPIT) (18). Peptide tandem mass spectra were acquired in a data-dependent manner over 16 h (8 MudPIT cycles) using a Thermo-Fisher LTQ. These spectra were queried against a UniProt rodent data base using SEQUEST (19), and resulting identifications were filtered and collated by protein using IDPicker (20).

SDS-PAGE and Autoradiography—L8 cells were incubated with ^{75}Se-labeled Sepp1 for 5 h in the presence or absence of 100 μM chloroquine. Cells were washed 3 times with PBS and lysed with PBS containing 0.2% Triton X-100. Aliquots of lysate were used for SDS-PAGE and for immunoaffinity purification of Sepp1. 0.5 ml of PBS, 0.2% Triton X-100 was added to 100 μl of lysate, and the mixture was incubated for 1 h with anti-mouse Sepp1 (9S4) conjugated with 50 μl of AminoLink beads. The beads were pelleted by centrifugation at 1500 *g* for 1 min, and the pellet was washed 3 times with 1 ml of PBS each time. The final pellet was incubated in SDS-PAGE sample buffer heated to 100 °C for 5 min and then centrifuged. The supernatant fraction collected was ~30 μl, and 25 μl was used for SDS-PAGE and autoradiography. 1 μl of ^{75}Se-labeled *Sepp1*$^{-/-}$ mouse serum

TABLE 2
Mass spectrometric identification of L8 cell Sepp1-binding proteins

Lrp1 peptides 18% coverage	
DCPDGSDEAPEICPQSK	SIHLSDER
NGDTCVTLLDLELYNPK	NLNAPVQPFEDPEHMK
VFFTDYGQIPK	NVIALAFDYR
SGFSLGSDGK	AGTSPGTPNR
DIFVTSK	IFFSDIHFGNIQQINDDGSGR
LYWVDAFYDR	TTIVENVGSVEGLAYHR
SERPPIFEIR	GWDTLYWTSYTTSTITR
MYDAQQQQVGTNK	ETVITMSGDDHPR
CININWR	AALSGANVLTLIEK
LDGLCIPLR	ILQEDFTCR
CSCYEGWVLEPDGESCR	DKSDEKPSYCNSR
TTLLAGDIEHPR	TACGVGEFR
DGILFWTDWDASLPR	FCSEAQFECQNHR
IEAASMSGAGR	CVAEALLCNGQDDCGDGSDER
ETGSGGWPNGLTVDYLEK	GCHVNECLSR
ILWIDAR	KLSGCSQDCEDLK
YDGSGHMEVLR	CLCVEGYAPR
TNTQPFDLQVYHPSR	AVTDEEPFLIFANR
VYWSDVR	QGLNNAVALDFDYR
NLFWTSYDTNKK	TGLSNPDGLAVDWVGGNLYWCDK
GPVGLAIDFPESK	IGMDGSGR
CNLDGSELEVIDTMR	ITWPNGLTVDYVTER
ATALAIMGDK	IYWADAR
LWWADQVSEK	EDYIEFASLDGSNR
ADGSGSVVLR	CACPTNFYLGGDGR
VYDESIQLEHEGTNPCSVNNGDCSQLCLPTSETTR	CDTEDDCGDHSDEPPDCPEFK
SCMCTAGYSLR	CDMDQFQCK
YVVISQGLDKPR	AEGSEYQVLYIADDNEIR
AITVHPEK	GIAIDWVAGNVYWTDSGR
LYWCDAR	IETAAMDGTLR
TGIGVQLK	LYWADAK
GTNVCAVANGGCQQLCLYR	HSLASTDEKR
EYAGYLLYSER	
ApoER2 peptides 22% coverage	
ELFVLPGEPR	IYSAHMDK
NPLSELPVVK	AIAVDPLR
TAQIGHVYPAR	GFMYWSDWGFQAK
CDGEEECPDGSDESK	QTLVSDNIEWPNGITLDLLSQR
ATCSSEECPAEK	VFWTDLENEAIFSANR
IGFECTCPAGFQLLDQK	YTCACPDTMWLGPDMK
CECHPGYEMDTLTK	SMNFDNPVYR
SPSLIFTNR	KTTEEEEEDELHIGR
IYWCDLSYR	LYWVDSK

was applied as a control. For the autoradiograph used to visualize selenoproteins in L8 cells, SDS-PAGE was performed using a 4–12% Bis-Tris NuPAGE Novex acrylamide gel as described previously (9). The dried gel was exposed to Kodak XAR film for 7 days before the film was developed.

RT-PCR—Cells rinsed with PBS or tissues pulverized under liquid nitrogen were treated with TRIzol Reagent. Bone marrow cells were obtained from femur and tibia (21). Total RNA was isolated according to the manufacturer's protocol. RNA concentration was determined by measurement of A_{260}. cDNA was made from total RNA by the High Capacity cDNA Reverse Transcriptase kit with 0.5 μg of total RNA per 20-μl reaction following the manufacturer's instructions. Quantitative PCR for gene expression was performed with 5 μl of diluted cDNA using Power SYBR Green PCR Master Mix with specific primer concentration at 250 nM in a total reaction volume of 20 μl. StepOnePlus Real-time PCR System and StepOne software V2.1 (Applied Biosystems) were used to collect and analyze data. Three replicates of each sample were amplified. For cell experiments, relative quantitation of RNA levels was determined by construction of relative standard curves. The control cDNA for these curves was made from total RNA isolated from cells treated with Universal Negative Control#1 siRNA. Relative standard curves were generated for each gene by serial 1:5 dilutions of the control cDNA to give a final dilution of 1:3125. cDNA from siRNA-treated cells (1:125 dilutions) were amplified simultaneously with control cDNA (1:125 dilution). Primers for amplification of rat Lrp1, apoER2, and cyclophilin A (Table 1) were used. Cyclophilin A served as the endogenous control. The target mRNA quantity in each siRNA-treated sample was determined from the relative standard curve (using sample CT values) and expressed in arbitrary units (relative quantitation). For tissue experiments, relative quantitation of RNA levels was determined by comparative CT reactions ($\Delta\Delta C_T$ analysis). Primers for amplification of mouse apoER2 and hypoxanthine phosphoribosyltransferase (Hprt) (Table 1) were used. Hypoxanthine phosphoribosyltransferase served as the endogenous control. The synthesized cDNA was diluted 1:125 before use. The target mRNA quantity in each tissue was expressed in arbitrary units (relative quantitation).

Statistics—Statistical comparisons between groups were made on an iMac using Prism 5 for Macintosh Version 5.00.2 software program (GraphPad Software, Inc.). Tukey's multiple comparison test was applied after analysis by 1-way ANOVA. Where appropriate, Student's t test was used to compare groups. Groups were considered to be significantly different when $p < 0.05$.

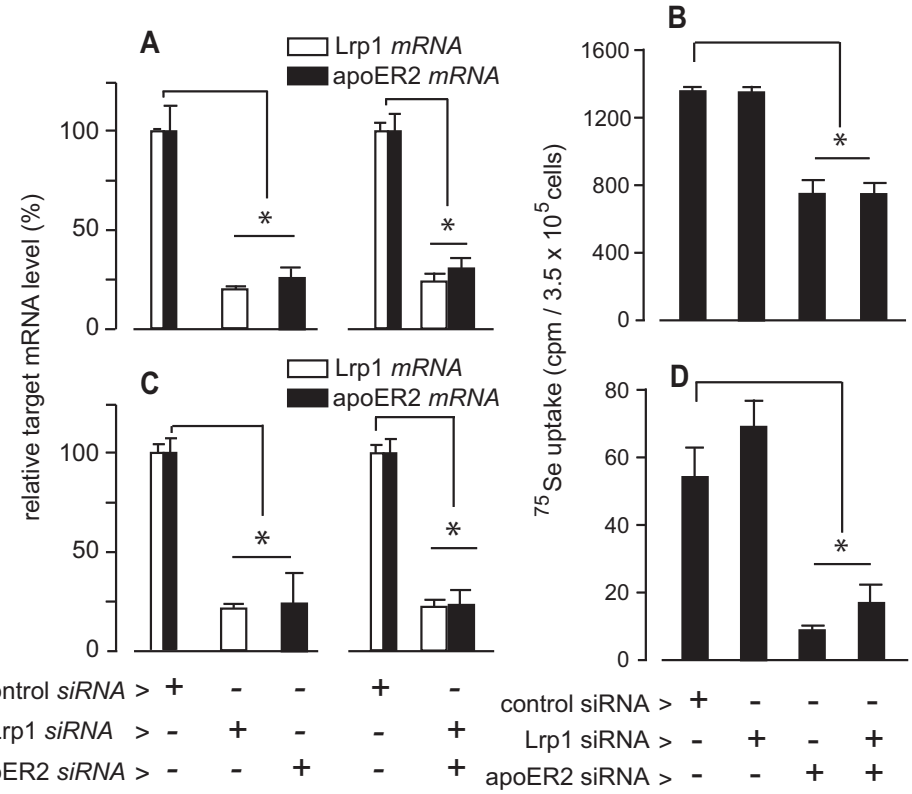

FIGURE 4. **Effect of knock down of receptors apoER2 and/or Lrp1 on ^{75}Se uptake from ^{75}Se-labeled Sepp1 by L8 and H9c2 cells.** *A*, L8 cells were cultured for 48 h with siRNA, and then relative mRNA levels were determined. Values are the means ($n = 3$) with 1 S.D. indicated by the *bracket*. The *asterisk* indicates values different from control siRNA ($p < 0.05$) by Student's *t* test. *B*, uptake in 5-h incubations of ^{75}Se by L8 cells with knockdown of receptors is shown. Values are the means of four experiments done in duplicate with 1 S.D. indicated by the *bracket*. *Asterisks* indicate that control was different ($p < 0.05$) from apoER2 and double knockdowns by Student's *t* test. *C*, H9c2 cells were cultured for 48 h with siRNA, and relative mRNA levels were determined. Values are the means ($n = 3$) with 1 S.D. indicated by the *bracket*. The *asterisk* indicates values different ($p < 0.05$) from control siRNA by Student's *t* test. *D*, shown is uptake in 5-h incubations of ^{75}Se by H9c2 cells with knockdown of receptors. Values are the means of four experiments done in duplicate with 1 S.D. indicated by the *bracket*. The *asterisk* indicates that control is different ($p < 0.05$) from apoER2 and double knockdowns by Student's *t* test.

RESULTS

Uptake of Sepp1 and Utilization of Its Selenium—Uptake of selenium from the culture medium and utilization of it for Gpx synthesis were studied in the rat myoblast L8 cell line. L8 cells took up ^{75}Se in a time-dependent manner from mouse *Gpx3*$^{-/-}$ serum that contained ^{75}Se-labeled Sepp1 as the only selenoprotein (Fig. 1*A*). We chose 5 h as the end point for subsequent ^{75}Se-Sepp1 uptake studies.

Inhibition of ^{75}Se uptake by unlabeled serum selenoproteins was assessed. Unlabeled *Gpx3*$^{-/-}$ serum, which contained Sepp1 (all isoforms), inhibited ^{75}Se uptake in relation to the amount of serum added (Fig. 1*B*), but serum from *Sepp1*$^{\Delta 240-361/\Delta 240-361}$ mice, which contained Gpx3 and Sepp1$^{\Delta 240-361}$ (the shortest isoform of Sepp1) as the only selenoproteins (6), did not inhibit uptake (Fig. 1*C*). These findings suggest that the presence of the C-terminal domain of Sepp1, which is not present in Sepp1$^{\Delta 240-361}$, is needed for the competition with ^{75}Se uptake from ^{75}Se-labeled "all isoform" Sepp1.

The ability of different selenium forms to support cell Gpx activity was assessed. Selenium sources were incubated for 24 h with selenium-deficient cells, and then Gpx activity of the cells was measured. Selenite supported Gpx activity with its maximum effect being reached at 100 nM (Fig. 2*A*), defining the maximal Gpx synthesis by the cells in 24 h. All-isoform Sepp1-containing serum that provided 83 nM medium selenium caused an increase in L8 cell Gpx activity from the basal 23% of that achieved with 100 nM selenite to 77% (Fig. 2*B*). Serum containing Gpx3 or Sepp1$^{\Delta 240-361}$ at comparable selenium concentrations did not increase Gpx activity, however. These results complement the inhibition results and indicate that one or more of the longer isoforms of Sepp1 supplied selenium to the cells for synthesis of Gpx enzymes.

Route of Sepp1 Uptake—Sepp1 is a heparin-binding protein (22). We evaluated the requirement of that property for ^{75}Se uptake by L8 cells. When heparin was added to the medium, ^{75}Se uptake from ^{75}Se-labeled Sepp1, but not from ^{75}Se-labeled selenite, was inhibited (Figs. 3, *A* and *D*). This result is compatible with heparin masking the heparin-binding sites of Sepp1 and preventing it from binding to HSPGs on the cell surface. Adding protamine, a compound that binds to heparin and to HSPGs, also inhibited ^{75}Se uptake from ^{75}Se-labeled Sepp1 (Fig. 3*B*). It had a slight effect on uptake of ^{75}Se from ^{75}Se-labeled selenite (Fig. 3*E*). Chlorate treatment of cells inhibits heparan sulfate maturation and depletes cell-surface HSPGs. Chlorate inhibited ^{75}Se uptake from ^{75}Se-labeled Sepp1 in a dose-dependent manner (Fig. 3*C*) but had only a slight effect on uptake of ^{75}Se from ^{75}Se-labeled selenite (Fig. 3*F*). Thus, it appears that the heparin binding properties of Sepp1 facilitate uptake of its selenium by L8 cells, suggesting that Sepp1 binding to HSPGs on the cell surface is required for its uptake by the cell.

We sought Sepp1 receptors in membranes of L8 cells by preparing a solubilized membrane fraction and passing it over a column with rat Sepp1 bound to the beads. The eluted fraction was analyzed by mass spectrometry. Table 2 shows that apoER2 peptides with 22% coverage of the apoER2 sequence and Lrp1 peptides with 18% coverage of its sequence were identified. Megalin peptides were not detected. This indicates that apoER2 and Lrp1, both members of the low density lipoprotein receptor family, were present in L8 cells and that they bound to the Sepp1 column. Thus, either of these receptors or both of them might be responsible for Sepp1 uptake.

We employed siRNA techniques to determine the role of these receptors in Sepp1 uptake. Cells were prepared in which each receptor was knocked down and in which both were knocked down simultaneously. Fig. 4*A* shows that the mRNAs were knocked down to less than 31% of control. Knockdown of Lrp1 had no effect on uptake of ^{75}Se from ^{75}Se-labeled Sepp1 (Fig. 4*B*). Knockdown of apoER2 inhibited ^{75}Se uptake to 56% that of the negative siRNA control, and knockdown of both receptors had the same effect as knockdown of apoER2 alone. This implicates apoER2 in facilitating uptake by L8 cells of the selenium supplied as Sepp1.

The same experimental protocol was carried out using H9c2 cells, a cell line derived from embryonic BD1X rat heart tissue. Fig. 4*C* shows that the mRNA of Lrp1 was knocked down to 22% that of control and that of apoER2 to 25%. Uptake of ^{75}Se from ^{75}Se-labeled Sepp1 was not decreased by knockdown of Lrp1, but it was decreased to 18% of control by knockdown of apoER2 and to 31% by knockdown of both receptors simultaneously (Fig. 4*D*). Thus, apoER2, but not Lrp1, mediated uptake of Sepp1 selenium in this line of cardiac myoblasts.

Endocytosis takes place by several pathways so we investigated the roles of clathrin-dependent and caveolin-dependent pathways. Fig. 5*A* shows that chlorpromazine inhibited uptake of ^{75}Se from ^{75}Se-labeled Sepp1, suggesting that the process is clathrin-dependent. Nystatin, an inhibitor of the caveolin-dependent pathway, had no (or minimal) effect (Fig. 5*B*). ApoER2 has been reported to be endocytosed by the clathrin-dependent pathway so this result was not unexpected (23).

Lysosomal Digestion of Sepp1—Ligands of low density lipoprotein receptors are usually delivered to lysosomes for digestion. Chloroquine and bafilomycin A1 both inhibit lysosome acidification and thereby prevent digestion of proteins inside the lysosome (16, 17). Figs. 6, *A* and *B*, show that adding these inhibitors to the medium blocked development of Gpx activity after the addition of serum containing Sepp1. The inhibitors did not prevent uptake of ^{75}Se from ^{75}Se-labeled Sepp1, however (Fig. 6*C*).

We examined L8 cells that had been cultured with ^{75}Se-labeled Sepp1 in the presence of chloroquine to determine whether they contained undigested Sepp1. Lysed cells and Sepp1 that had been immunoaffinity-purified from lysed cells were subjected to SDS-PAGE, and ^{75}Se was detected by autoradiography (Fig. 6*D*). Immunoaffinity purification was carried out with the monoclonal antibody 9S4 that recognizes an epitope in the N-terminal domain of Sepp1. *Lane 5* represents serum containing ^{75}Se-labeled Sepp1, included to indicate the migration of the ^{75}Se-labeled Sepp1 that had been added to the culture medium. Chloroquine addition to the medium caused accumulation of apparently undigested ^{75}Se-labeled Sepp1 in cells (compare *lane 2* with *lane 1*). In the absence of chloroquine (*lane 3*) the only ^{75}Se-labeled Sepp1 form detected that bound to the antibody 9S4 was a fragment that migrated at 18 kDa, but in the presence of chloroquine (*lane 4*) the only ^{75}Se-labeled Sepp1 form that was visible migrated near the undigested serum ^{75}Se-labeled Sepp1 (*lane 5*). These results indicate that lysosomal digestion is necessary for utilization of the selenium in endocytosed Sepp1.

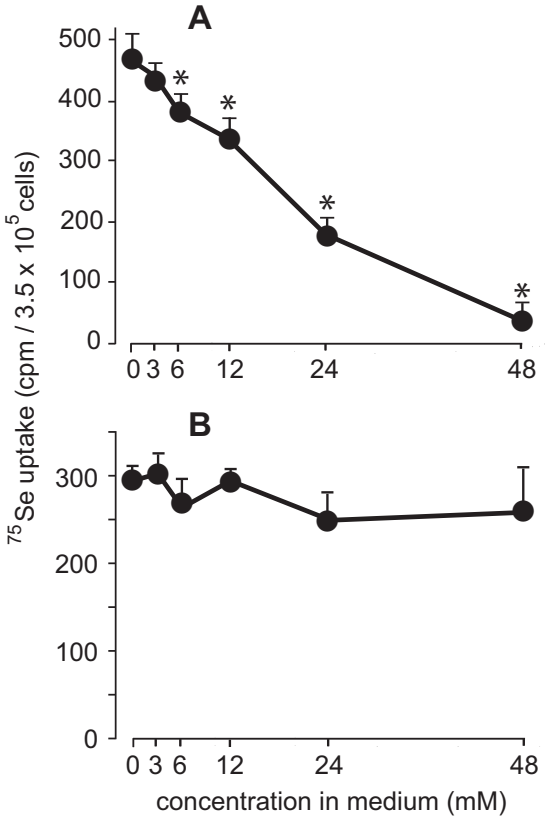

FIGURE 5. **Effect of inhibiting clathrin and caveolin endocytosis pathways on the uptake of ^{75}Se from ^{75}Se-labeled Sepp1.** L8 cells were incubated for 30 min in serum-free medium containing the indicated concentrations of chlorpromazine (*A*) or nystatin (*B*) before serum that contained ^{75}Se-labeled Sepp1 was added. After 5 h, cells were washed, and ^{75}Se was determined in them. Values are the means ($n = 4$) with 1 S.D. indicated by the *bracket*. *Asterisks* indicate values significantly different ($p < 0.05$) from the initial value by Tukey's multiple comparison test applied after one-way ANOVA of the groups.

ApoER2 mRNA Expression in Muscle and Other Tissues—ApoER2 mRNA is expressed at high levels in testis and brain (24). The results in Table 2 and Fig. 4 show that the rat L8 and H9c2 myoblast cell lines express apoER2 also. We carried out RT-PCR of several adult mouse tissues to determine the relative expression of apoER2 mRNA in them (Fig. 7). Expression by testis, bone marrow, placenta, brain, and thymus was greater than by spleen, quadriceps, and heart. Expression by quadriceps muscle was 1.4% that of expression by brain and 0.07% of expression by testis. ApoER2 mRNA was questionably present in kidney and liver. These results show that apoER2 is widely expressed in the body.

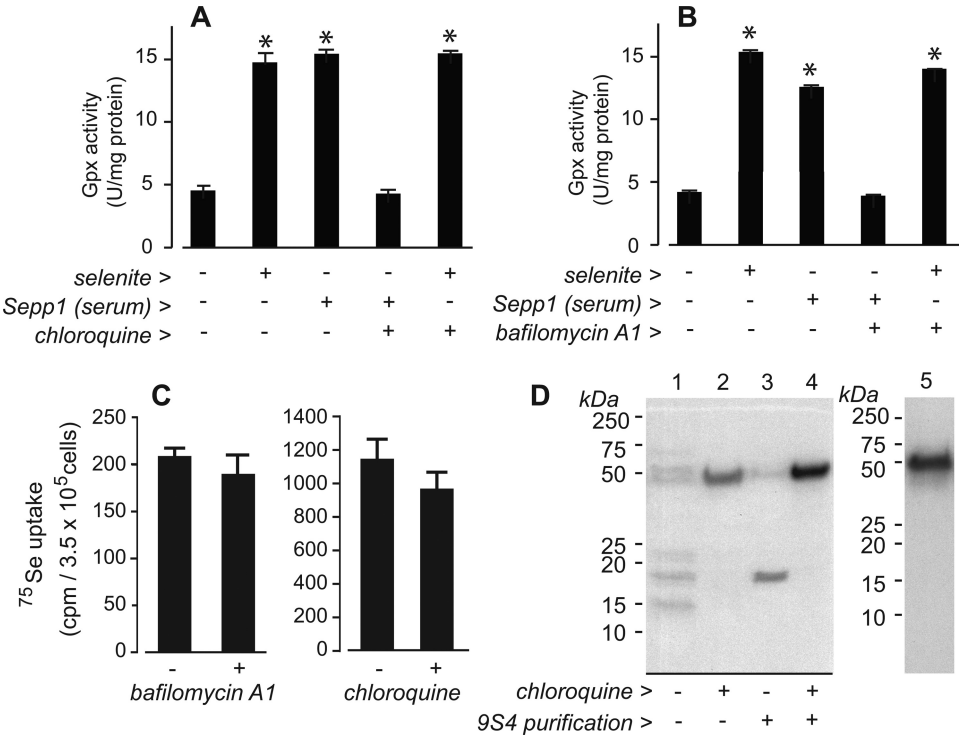

FIGURE 6. **Requirement of lysosomal function for L8 cell utilization of Sepp1 selenium.** *A*, shown is the effect of 100 μM chloroquine on Gpx activity in L8 cells cultured for 24 h with *Gpx3*$^{-/-}$ serum containing Sepp1 (98 nM selenium) or with selenite (100 nM selenium). *B*, shown is the effect of 100 nM bafilomycin A1 on Gpx activity in L8 cells cultured with *Gpx3*$^{-/-}$ serum containing Sepp1 (98 nM selenium) or with selenite (100 nM selenium). *C*, ^{75}Se uptake by L8 cells with bafilomycin A1 (100 nM) or chloroquine (100 μM) added to the medium is shown. Values in *A–C* are the means ($n = 3$ experiments carried out on duplicate plates) with 1 S.D. indicated by the *bracket*. Treated values were not significantly different from the control values ($p < 0.05$) by Student's *t* test. *D*, shown is the effect of chloroquine addition on Sepp1 form in L8 cells. L8 cells were incubated for 5 h with serum containing ^{75}Se-labeled Sepp1 with or without 100 μM chloroquine. After washing, cells were lysed with SDS-PAGE buffer. Whole-cell lysates were subjected to SDS-PAGE and autoradiography (*lanes 1* and *2*) or to purification of Sepp1 using 9S4 antibody before the eluate was subjected to SDS-PAGE and autoradiography (*lanes 3* and *4*). *Lane 5* shows an autoradiogram of 1 μl serum from a *Gpx3*$^{-/-}$ mouse that had been injected with ^{75}Se-labeled selenite.

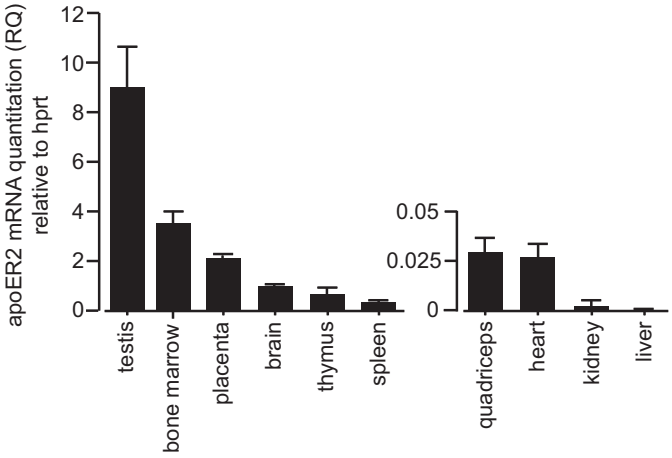

FIGURE 7. **Relative abundance of apoER2 mRNA in tissues of mice fed diet supplemented with 0.25 mg of selenium/kg as selenite.** Total RNA was extracted from designated tissues and used for cDNA synthesis and real-time PCR analysis. Levels of each tissue RNA are shown relative to hypoxanthine phosphoribosyltransferase. The brain value was set as 1, and the quantitation of each tissue is relative to it. Values are the means ($n = 3$) with the *bracket* indicating 1 S.D. The *panel on the right* shows tissues with very low relative mRNA expression (less than 3% of brain). *TQ*, relative quantitation.

DISCUSSION

The results presented here demonstrate that L8 muscle cells take up Sepp1 and utilize its selenium for selenoprotein synthesis. The uptake was inhibited by interference with binding to HSPGs and by knockdown of apoER2. After uptake of Sepp1 by the cell, utilization of its selenium required lysosomal digestion. ApoER2 mRNA expression was shown to occur in many tissues. These findings outline the process by which Sepp1 supplies selenium to an individual cell and have implications for the supply of selenium to cells throughout the body.

L8 cells take up selenium from all-isoform Sepp1 (Fig. 1). Neither the shortest isoform of Sepp1, Sepp1$^{\Delta 240-361}$, nor Gpx3 inhibits that uptake or provides selenium to the cells (Figs. 1 and 2). Thus, of the plasma selenoproteins, only one or more of the longer Sepp1 isoforms is (are) taken up. None of these putative mouse isoforms has been characterized, but all of them would contain some of the C-terminal domain, implying that recognition of the selenium-rich C-terminal domain of Sepp1 is necessary for the uptake. This *in vitro* finding complements observations *in vivo* that apoER2-mediated endocytosis of Sepp1$^{\Delta 240-361}$ does not occur in the testis or placenta.[3,4]

Another requirement for uptake of Sepp1 appears to be binding to HSPGs (Fig. 3). Receptor-mediated processes often involve binding of the ligand to cell surface HSPGs (25). Such binding can serve as a reservoir for the ligand and can facilitate interaction of the ligand with its receptor. Heparin-binding sites have been identified only in the N-terminal domain of Sepp1 (22), but C-terminal fragments of human Sepp1 have

[3] R. F. Burk, G. E. Olson, K. E. Hill, V. P. Winfrey, A. K. Motley, and S. Kurokawa, manuscript in preparation.
[4] R. F. Burk, G. E. Olson, K. E. Hill, and V. P. Winfrey, unpublished observation.

also been shown to bind to heparin (26). Thus, binding of Sepp1 to cell-surface HSPGs might occur through either the N-terminal domain or the C-terminal domain. This leaves open the possibility that any Sepp1 form that contains the selenium-rich C-terminal domain could be taken up by the mechanism under study. However, we presented to our cells only forms that contained the N-terminal domain so our results do not allow us to reach the conclusion that the N-terminal domain is not needed for uptake by L8 cells. Other possibilities remain, including that the HSPG interaction takes place through the N-terminal domain and the apoER2 binding through the C-terminal domain.

Receptor-mediated uptake of Sepp1 has been previously observed in only a few tissues, and those observations were made *in vivo*. ApoER2 facilitates Sepp1 uptake from the circulation by testis and brain (9, 10), and megalin facilitates Sepp1 uptake by proximal convoluted tubule cells from the urinary filtrate (11). There is evidence that these two receptors mediate uptake of different forms of Sepp1. Megalin mediates Sepp1 uptake in $Sepp1^{\Delta 240-361/\Delta 240-361}$ mice, and apoER2 does not (11). This indicates that the megalin uptake process, unlike that of apoER2, does not require the presence of the Sepp1 C-terminal domain. Also, the Sepp1 forms in urinary filtrate that are taken up by megalin appear to be smaller than the forms in the systemic circulation (27). Taken together, these observations suggest that apoER2 uptake of Sepp1 from the systemic circulation has the purpose of acquiring selenium for the cell via the selenium-rich C-terminal domain, whereas megalin uptake, which is from the urinary filtrate, has the 2-fold purpose of scavenging selenium-containing fragments of Sepp1 to prevent their loss in the urine and of supplying selenium to the proximal convoluted tubule cells for synthesis of Gpx3 (28).

Despite its binding to a Sepp1 column, knockdown of Lrp1 did not impair uptake of Sepp1 by muscle cells (Fig. 4). Thus, apoER2 is the only receptor presently known to mediate endocytosis of Sepp1 from the systemic circulation. Our demonstration that apoER2 is expressed in a number of tissues (Fig. 7) allows the speculation that apoER2-mediated endocytosis of Sepp1 occurs in many tissues of the body. Regulation of Sepp1 uptake by each tissue might be determined by expression of apoER2 by the tissue (Fig. 7) and/or by other factors, such as binding to cell-surface HSPGs.

Based on the results presented here, we postulate that apoER2-mediated endocytosis of Sepp1 ensures the supply of selenium to muscle cells (and to other cells that express this receptor) under selenium deficiency conditions, just as it has been shown to do in the brain (10). It has not been possible to test this directly because the production of selenium deficiency in $apoER2^{-/-}$ mice leads rapidly to neurological dysfunction and death before selenium deficiency can be established in the whole animal. However, comparison of tissue selenium concentrations in wild-type mice that were severely selenium-deficient with concentrations in selenium-replete mice revealed that retention in brain was 56%, testis was 22%, muscle was 15%, kidney was 9%, and liver was 3% (4). Fig. 7 shows that brain and testis had relatively high expressions of apoER2 mRNA. Muscle expressed a significant amount of apoER2 mRNA, but kidney and liver did not. Kidney, however, expresses megalin, and that might account for its retention of more selenium than liver. These correlations between selenium retention and apoER2 mRNA expression support a role for apoER2 in maintaining selenium supply to many tissues under conditions of selenium deficiency.

In conclusion, the finding that apoER2 mediates uptake of Sepp1 by two muscle cell lines and the recognition that many tissues express apoER2 suggest that Sepp1 uptake by apoER2-mediated endocytosis is responsible for maintaining the physiological distribution of selenium in the body under varying conditions of selenium supply.

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