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Selenophosphate synthetase 2 is essential for selenoprotein biosynthesis

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Selenophosphate synthetase (SelD) generates the selenium donor for selenocysteine biosynthesis in eubacteria. One homologue of SelD in eukaryotes is SPS1 (selenophosphate synthetase 1) and a second one, SPS2, was identified as a selenoprotein in mammals. Earlier *in vitro* studies showed SPS2, but not SPS1, synthesized selenophosphate from selenide, whereas SPS1 may utilize a different substrate. The roles of these enzymes in selenoprotein synthesis *in vivo* remain unknown. To address their function *in vivo*, we knocked down SPS2 in NIH3T3 cells using small interfering RNA and found that selenoprotein biosynthesis was severely impaired, whereas knockdown of SPS1 had no effect.

Transfection of SPS2 into SPS2 knockdown cells restored selenoprotein biosynthesis, but SPS1 did not, indicating that SPS1 cannot complement SPS2 function. These *in vivo* studies indicate that SPS2 is essential for generating the selenium donor for selenocysteine biosynthesis in mammals, whereas SPS1 probably has a more specialized, non-essential role in selenoprotein metabolism.

Key words: RNA interference, selenocysteine, selenocysteine synthesis, selenoprotein, selenoprotein synthesis, selenophosphate synthetase.

INTRODUCTION

Selenocysteine (Sec) is the 21st amino acid in the genetic code [1,2] and, unlike other amino acids, the biosynthesis of selenocysteine occurs on its tRNA [3,4]. Selenocysteine tRNA is initially aminoacylated with serine by seryl-tRNA synthetase and is therefore designated selenocysteine tRNA^{[Ser]Sec}. Although the biosynthetic pathway of selenocysteine was established in eubacteria in the early 1990s [5], little was known about the biosynthesis of this amino acid in eukaryotes and archaea until recently. The biosynthetic pathway in eukaryotes and archaea was found to have an extra step compared with eubacteria and proceeds from seryl-tRNA^{[Ser]Sec} to phosphoseryl-tRNA^{[Ser]Sec}, which is catalysed by phosphoseryl-tRNA^{[Ser]Sec} kinase [6], and then to selenocysteyl-tRNA^{[Ser]Sec}, which is catalysed by selenocysteine synthase [7,8]. In eubacteria, the pathway proceeds from seryl-tRNA^{[Ser]Sec} to selenocysteyl-tRNA^{[Ser]Sec} and a single selenocysteine synthase, designated SelA (*Escherichia coli* selenocysteine synthase), carries out this step [5]. The active donor of selenium that makes selenocysteyl-tRNA^{[Ser]Sec} from the intermediate generated by SelA in bacteria is monoselenophosphate [9], which is synthesized by selenophosphate synthetase from selenite and ATP. Selenophosphate appears to be the universal donor in the biosynthesis of this amino acid [8,9]. Selenophosphate synthetase is designated SelD in bacteria and SPS2 (selenophosphate synthetase 2) in mammals. Interestingly, in mammals, SPS2 is a selenoprotein, which suggests that SPS2 can be an auto-regulator as well as a regulator of selenoprotein synthesis as a whole [10,11].

In addition to SPS2, another protein, SPS1, was also originally thought to have a role in selenophosphate synthesis [10–13]. Complementation studies with SPS1 and SPS2 in *E. coli* cells, however, suggested that these proteins might have different roles.

Initially, the selenocysteine to cysteine mutant of SPS2 was found to have low enzyme activity [10,11,14] and this mutant form also complemented SelD in *E. coli* cells transfected with the mammalian mutant protein [11,14]. SelD⁻ *E. coli* cells could also be complemented with SPS1 and SPS2, but the results suggested that SPS2 was involved in the *de novo* synthesis of selenophosphate, whereas SPS1 may have a role in recycling selenocysteine by a selenium salvage system [15]. We recently demonstrated that SPS2 synthesizes selenophosphate *in vitro* and that this compound is the active donor for making selenocysteine; we also showed that SPS1 does not synthesize selenophosphate [8]. However, these studies were done *in vitro* and, most importantly, the phenotypic analysis of mammalian cells in which SPS1 and SPS2 have been targeted for removal, and the complementation of such cells with SPS1 and SPS2, have not been carried out. In the present study, we therefore examined the effect of the knockdown of SPS1 and SPS2 in NIH3T3 cells on selenoprotein synthesis and have complemented SPS2 knockdown cells with SPS1 and SPS2. The results of the present study demonstrate that the role of SPS2 is to provide the active selenium donor for selenocysteine synthesis in mammals and that SPS1 must have another role in selenium metabolism, which does not involve monoselenophosphate synthesis.

EXPERIMENTAL

Materials

[⁷⁵Se]Selenium (specific activity 1000 Ci/mmol) was purchased from the Research Reactor Facility (University of Missouri, Columbia, MO, U.S.A.), in the form of selenious acid and

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx1, glutathione peroxidase 1; HRP, horseradish peroxidase; mSPS, mouse selenophosphate synthetase; mSPS2-Cys, mSPS2 containing a selenocysteine (UGA) to cysteine (UGC) mutation; RT, reverse transcription; SelA, *Escherichia coli* selenocysteine synthase; SelD, *Escherichia coli* selenophosphate synthetase; SelT, selenoprotein T; siRNA, small interfering RNA; SPS, selenophosphate synthetase; TR1, thioredoxin reductase 1.

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neutralized with NaOH before use. [α - 32 P]dCTP (specific activity approx. 6000 Ci/mmol) and [α - 32 P]ATP (specific activity approx. 3000 Ci/mmol) were from NEN Corporation, and 3 H-serine (specific activity 29 Ci/mmol) and Hybond Nylon N⁺ membranes were from Amersham Biosciences. NuPage 10% (w/v) polyacrylamide gels, PVDF membranes, Superscript II reverse transcriptase, cell culture FBS (fetal bovine serum), DMEM (Dulbecco's modified Eagle's medium), LipofectamineTM 2000, TRIzol[®] reagent and Hygromycin B were purchased from Invitrogen. DNA polymerase I large Klenow fragment was from New England BioLabs, pET and pTriEx-4-Hygro vectors were from Novagen and SuperSignal West Dura Extended Duration Substrate from Pierce. Anti-His-tag antibodies were obtained from Qiagen, HRP (horseradish peroxidase)-conjugated secondary antibodies from Sigma and QuikChange[®] II Site-Directed Mutagenesis kits from Stratagene. All other chemicals and reagents were obtained commercially and were of the highest grade available.

Mammalian cell culture and transfection

Mouse embryonic fibroblast (NIH3T3) cells were obtained from A.T.C.C. and cultured in DMEM supplemented with 10% (v/v) FBS. Transfections were carried out using LipofectamineTM 2000 according to the manufacturer's instructions. The medium of the cells grown in the presence of selenium was supplemented with 200 nM sodium selenite or selenocysteine.

Generation of SPS siRNA (small interfering RNA) constructs

The Tet-on U6 vector used for expressing siRNA constructs was prepared using the pU6-m4 Hygro vector [16] as the backbone. Two O2-type *tet* operators [17] were placed flanking the TATA-box of the U6 promoter using QuikChange II site-directed mutagenesis. This modified vector was designated as pU6-TetO4m4.

For knocking down the expression of *Sps1* (NM_175400) and *Sps2* (NM_009266), five separate 19 nt sequences (*Sps1*: nucleotides 119–137, 418–436, 694–712, 1179–1197 and 1396–1414; *Sps2*: nucleotides 567–585, 830–848, 1265–1283, 1684–1702 and 1852–1870) were selected from each cDNA as being unique to the respective gene using the online service, siDESIGN, of Dharmacon Research. The corresponding siRNA constructs within the pU6-TetO4m4 vector were prepared as described previously [16]. The sequences of each SPS siRNA construct were verified by sequencing, and the five constructs encoding the SPS siRNA constructs and the pU6-TetO4m4 empty vector control were transfected separately into NIH3T3 cells. After stabilizing the transfected cells with 0.8 mg/ml of hygromycin, *Sps1* and *Sps2* mRNA levels were determined by Northern blot hybridization as described below. On the basis of the knockdown of mRNA levels, the best siRNA target sequence for each mRNA was identified as 5'-GACGTAGAGTTGGCATAACC-3' (nucleotides 694–712) in *Sps1* and 5'-GGAACGAGAGAAGGTGACA-3' (nucleotides 567–585) in *Sps2* and these SPS siRNA constructs, designated siSPS1 and siSPS2 respectively, were used in subsequent experiments. The control vector, pU6-TetO4m4, was designated pU6Tet control and it consisted of the same construct minus the target sequence.

Northern blot analyses

Total RNA was isolated from various transfected cells using TRIzol[®] reagent. Equal amounts of RNA (15 μ g) from the different cells was loaded onto 1.2% (w/v) agarose gels with 4%

(v/v) formaldehyde and resolved by electrophoresis. The RNA was then transblotted onto a nylon membrane, the membrane hybridized with the appropriate [α - 32 P]dCTP-labelled probe and analysed with a PhosphorImager for Northern blot analysis as described previously [18]. The SPS1, SPS2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probes were generated by either PCR or RT (reverse transcription)-PCR using Superscript II reverse transcriptase and Universal Reference RNA [18]. The sizes of all the probes generated by RT-PCR were in the 500–1200 bp range. To detect an array of mRNAs on the same membrane, the membranes, after an initial probing for *Sps1* mRNA, were stripped in boiling buffer [0.1 \times SSC (1 \times SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS] before being hybridized with a second probe, using successively labelled SPS1, SPS2 or GAPDH cDNA probes. The hybridized membranes were exposed to Phosphor screens and the screens were then scanned using a Storm Image System (Molecular Dynamics).

SPS gene cloning

Mouse *Sps1* (NM_175400) and *Sps2* (NM_009266), and *E. coli SelD* (M30184) cDNA were amplified using RT-PCR as described previously [8], and then cloned into the pTriEx-4 Hygro vector for expression in mammalian cells. The SPS2-Cys [SPS2 containing a selenocysteine (UGA) to cysteine (UGC) mutation] mutant was generated as described previously [8]. Each gene contained a His-tag on either the N- or C-terminus for easy detection by Western blot analyses. To knock-in the selenocysteine (UGA) to cysteine (UGC) mutant of mSPS2 (mouse SPS2), four degenerate base mutations at nucleotides 570 (A \rightarrow G), 573 (A \rightarrow C), 576 (G \rightarrow A) and 582 (G \rightarrow C) in the 3'-position of each codon within the siRNA target region that still encoded the same amino acids of mSPS2 were carried out using QuikChange II site-directed mutagenesis. The sequence was confirmed by DNA sequencing.

Selenoprotein labelling

To label the selenoprotein population in NIH3T3 cells, the cells were grown overnight in DMEM with 10% (v/v) FBS and 50 μ Ci/ml of [75 Se]. After incubation, the labelled cells were harvested, then washed in PBS, resuspended in 1 \times SDS loading buffer [4 \times :1 M Tris/HCl, pH 8.5, 8% (v/v) SDS, 40% (v/v) glycerol, 1.6 mM EDTA, 0.075% Serva Blue G250 and 0.025% Phenol Red] and electrophoresed on NuPage 10% (w/v) polyacrylamide gels (along with standard molecular mass markers for assessing labelled selenoprotein sizes). After electrophoresis, the gels were stained with Coomassie Blue, then dried before being exposed to Phosphor screens, and the screens were scanned using a Storm Image (PhosphorImager) system as described previously [18].

Western blot analyses

For Western blot analyses, NIH3T3 cells were harvested, resuspended in 1 \times SDS loading buffer and the lysates were ultrasonicated using a Sonic Dismembrator 550 (Fisher Scientific) on power setting 5, with two 10 s pulses with a 2 min pause inbetween to shear the genomic DNA. Aliquots (50 μ g) of proteins from cell lysates were electrophoresed on NuPage 10% (w/v) polyacrylamide gels and transferred to PVDF membranes as described previously [18]. The membranes were incubated with rabbit-anti-mouse GPx1 (glutathione peroxidase 1; diluted 1:1000), SelT (selenoprotein T; diluted 1:2000), TR1

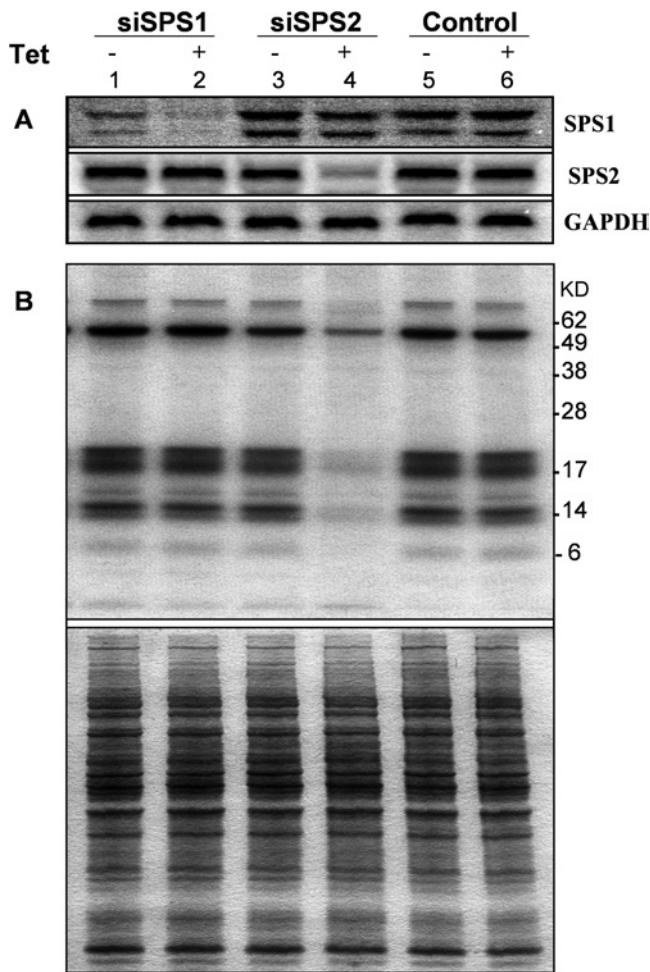


Figure 1 Knockdown of SPS1 and SPS2 in NIH3T3 cells

NIH3T3 cells were stably transfected with the siSPS1, siSPS2 and pU6Tet control constructs, and the cells grown in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of doxycycline for 3 days. **(A)** Northern blot analysis of RNA was performed on extracts of siSPS1 (lanes 1 and 2) and siSPS2 (lanes 3 and 4) knockdown cells, and pU6Tet control cells (lanes 5 and 6). The level of GAPDH mRNA was analysed as a loading control. **(B)** Upper panel, [⁷⁵Se]-labelled cell extracts were examined to evaluate selenoprotein synthesis in siSPS1 (lanes 1 and 2) and siSPS2 (lanes 3 and 4) knockdown cells, and control cells (lanes 5 and 6). Molecular masses are shown in kDa (KD). Coomassie Blue staining of the gel is shown as a protein loading control in the lower panel.

(thioredoxin reductase 1; diluted 1:1000), β -tubulin (diluted 1:2000) or anti-His-tag (diluted 1:2000) antibodies, and HRP-conjugated secondary antibodies (diluted 1:15000) were then applied. The membranes were then washed with Tris-buffered saline with Tween 20 (30 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1 % Tween 20), incubated in SuperSignal West Dura Extended Duration Substrate and exposed to X-ray film as described previously [18].

RESULTS

siSPS1 and siSPS2 target their respective mRNAs

Northern blot analysis was used to document the effect of siSPS1 and siSPS2 by reducing the steady-state levels of the corresponding gene expression following transfection of NIH3T3 cells with the appropriate construct. Both SPS siRNAs knocked down their corresponding mRNA in NIH3T3 cells effectively

when the constructs were induced with 5 μ g/ml of doxycycline for 3 days (Figure 1A, lanes 2 and 4). There did not appear to be cross-knockdown of mRNA between the two SPS siRNAs. Some leakiness apparently occurred in siSPS1 expression without induction with doxycycline in the knockdown of SPS1, as the amount of mRNA was less than that in the control (Figure 1A, compare lane 1 with lanes 5 and 6).

Effect of siSPS1 and siSPS2 on selenoprotein expression

To examine the effect of the siSPS knockdown constructs on selenoprotein expression, NIH3T3 cells stably transfected with the siRNA or control constructs were grown in the presence or absence of 5 μ g/ml of doxycycline for 3 days and then labelled with 50 μ Ci/ml of [⁷⁵Se] for 16 h (Figure 1B). Clearly, inducing siSPS2 had a pronounced effect on selenoprotein synthesis (Figure 1B, lane 4), whereas inducing siSPS1 had no apparent effect (Figure 1B, lane 2). Protein loading of each lane was shown by Coomassie Blue staining (Figure 1B, lower panel). We also labelled cells stably transfected with another siSPS2 construct that was found to be effective in knocking down SPS2 mRNA (target region, nucleotides 1684–1702). This construct manifested the same effect on selenoprotein synthesis when grown under identical conditions as the corresponding construct shown in Figure 1 (results not shown), which provided strong evidence that the phenotype resulting from the siSPS2 construct used throughout this study is not the result of off-target effects.

We next examined whether selenoprotein synthesis could be rescued in SPS2 knockdown cells by SPS1, SPS2 and/or SelD. NIH3T3 cells that were stably transfected with the siSPS2 construct were grown in the presence or absence of doxycycline for 3 days and then transfected with expression vectors encoding mSPS1, mSPS2, mSPS2-Cys, SelD or the pTri-Ex4 control vector. Several mutations were made within the siRNA target site in the mSPS2 and mSPS2-Cys constructs, as described in the Experimental section, to circumvent being targeted for removal in the siSPS2 stably transfected cells. After 48 h of transfection with the expression constructs, the cells were labelled with 50 μ Ci/ml of [⁷⁵Se] for 16 h. mSPS2, mSPS2-Cys and SelD restored selenoprotein synthesis, whereas mSPS1 did not (Figure 2A). The protein loading of each lane was shown by Coomassie Blue staining (Figure 2A, lower panel). Western blot analysis using the anti-His-tag antibody showed that the expression levels of the transfected mSPS1 and mSPS2-Cys were many times higher than the expression of transfected mSPS2 and SelD in siSPS2 stably transfected cells (Figure 2B, lanes 1 and 2). However, mSPS2 and SelD were expressed in similar amounts in cells transfected with the corresponding expression constructs (Figure 2B, lanes 3 and 4). Even the elevated expression of mSPS1 could not compensate for the loss of endogenous SPS2 in mammalian cells.

Effect of selenium, siSPS1 and siSPS2 on selenoprotein expression

To assess the effect of the SPS siRNA constructs on selenoprotein expression and, in addition, to examine the role that selenium may have on selenoprotein expression in the knockdown cells, NIH3T3 cells stably transfected with either the siSPS1, siSPS2 or control construct were grown in medium with or without selenite or selenocysteine, and three individual selenoproteins were examined by Western blot analysis (Figure 3). TR1, GPx1 and SelT were poorly expressed in SPS2 knockdown cells and there did not appear to be any change in the expression of these selenoproteins in cells grown in the presence or absence of selenite or in selenocysteine-supplemented medium, with the possible

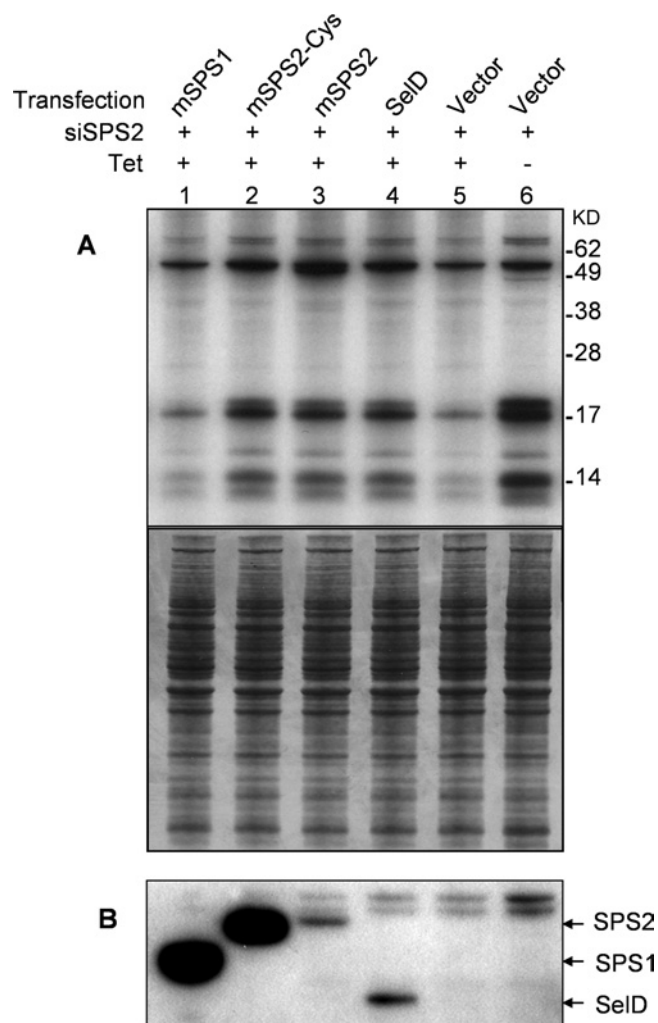


Figure 2 Rescue of selenoprotein synthesis in SPS2 knockdown cells

NIH3T3 cells were stably transfected with the siSPS2 construct and grown in the presence (lanes 1–5) or absence (lanes 6) of doxycycline for 3 days and then transiently transfected with the mSPS1 (lanes 1), mSPS2-Cys (lanes 2), mSPS2 (lanes 3), SelD (lanes 4) or pU6Tet control constructs (lanes 5 and 6). The cells, 48 h after transfection, were labelled overnight with [⁷⁵Se] and cell lysates electrophoresed on polyacrylamide gels for radiography or Western blot analysis as described in the Experimental section. (A) Upper panel, [⁷⁵Se] labelling of transfected NIH3T3 cells is shown from each extract as indicated. Molecular masses are shown in kDa (KD). Coomassie Blue staining of the gel is shown in the lower panel as a protein loading control. (B) Western blot analysis of His-tagged proteins from each transfected NIH3T3 cell line is shown as indicated. The amount of extract loaded onto gels in lanes 1–2 was 10-fold less than in lanes 3–6.

exception of SelT, which manifested a slight induction in the presence of either selenium source (Figure 3, lanes 4–6). TR1 was expressed in SPS1 knockdown cells grown in medium without a selenium source and the level of this protein was elevated in the presence of selenite or selenocysteine (Figure 3, lanes 1–3). GPx1 and SelT were poorly expressed in the absence of selenium and the levels of these selenoproteins were increased in response to selenite or selenocysteine. The pattern of expression of each selenoprotein was similar in the control cells to that observed in the SPS1 knockdown cells, with the exception of TR1, which appeared to be slightly higher in the presence and absence of selenium or selenocysteine in the SPS1 knockdown cells (Figure 3, compare lanes 7–9 with lanes 1–3).

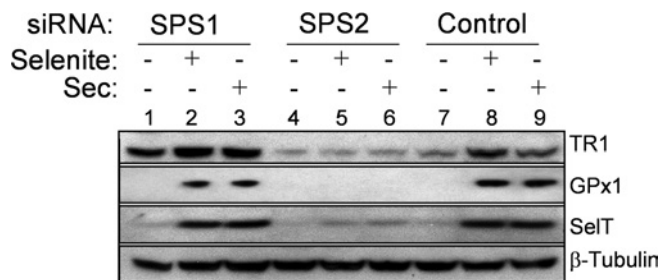


Figure 3 Western blot analysis of siSPS1 and siSPS2 knockdown cells

NIH3T3 cells were stably transfected with the siSPS1 construct (lanes 1–3), the siSPS2 construct (lanes 4–6) or the pU6Tet control construct (lanes 7–9), grown in the presence of doxycycline for 3 days and then grown either in the absence (lanes 1, 4 and 7) or presence of 200 nM selenite (lanes 2, 5 and 8) or 200 nM selenocysteine reduced from selenocysteine (lanes 3, 6 and 9) for an additional 2 days. The cell lysates of each transfected cell line were used for Western blotting of TR1, GPx1 and SelT as described in the Experimental section. Western blot analysis of β-tubulin was used as a loading control.

Effect of siSPS1 on selenoprotein turnover

To assess whether the knockdown of SPS1 may affect the turnover of selenoproteins, NIH3T3 cells stably transfected with the siSPS1 construct or the control vector were grown in the presence of 5 μg/ml of doxycycline for 3 days and then labelled with 50 μCi/ml of [⁷⁵Se] for 16 h. The cells were washed to remove the remaining [⁷⁵Se] in the medium and then expanded in fresh medium in 6-well plates with doxycycline, and the cells harvested at 0, 8, 24, 32, 48, 56 and 72 h. The decline of [⁷⁵Se] label in selenoproteins over the 72 h period is shown in Figure 4. There was no significant difference observed in the loss of [⁷⁵Se] in selenoproteins in the SPS1 knockdown cells and in the control cells. This result indicates that the targeted removal of SPS1 did not affect recycling of [⁷⁵Se] in selenoprotein synthesis in NIH3T3 cells.

DISCUSSION

The characterization of the pair of SPSs in mammals remained enigmatic in spite of numerous previous studies as discussed in the Introduction. We have demonstrated recently that SPS2, but not SPS1, can synthesize selenophosphate *in vitro* [8]. Furthermore, this earlier study used selenophosphate as the substrate in the biosynthesis of selenocysteine to demonstrate unequivocally that this compound is the active selenium donor (see [8] and references therein). The emphasis of the present study was to assess the consequences of removal of SPS1 and SPS2 in a cell culture model. The reduction in the expression of SPS2 inhibited selenoprotein expression, whereas the reduction in SPS1 expression had no effect. In addition, SPS2, as well as *E. coli* SelD, but not SPS1, complemented SPS2 knockdown in cells by restoring selenoprotein synthesis. It would seem that SPS2-generated selenophosphate must therefore be the sole donor for selenocysteine synthesis from selenite in mammals, since SPS2 and SelD synthesize selenophosphate and these proteins complement SPS2-deficient cells.

Selenium is known to regulate selenoprotein expression (reviewed in [1,2]) and some selenoproteins are more responsive to changes in selenium status than others. For example, GPx1 is poorly expressed when selenium levels are low and elevates dramatically when selenium levels are increased. In liver, levels of GPx1 may change by over 100-fold in response to selenium status. TR1 is also regulated by selenium, but its level changes only slightly. These data are consistent with the idea that there is a

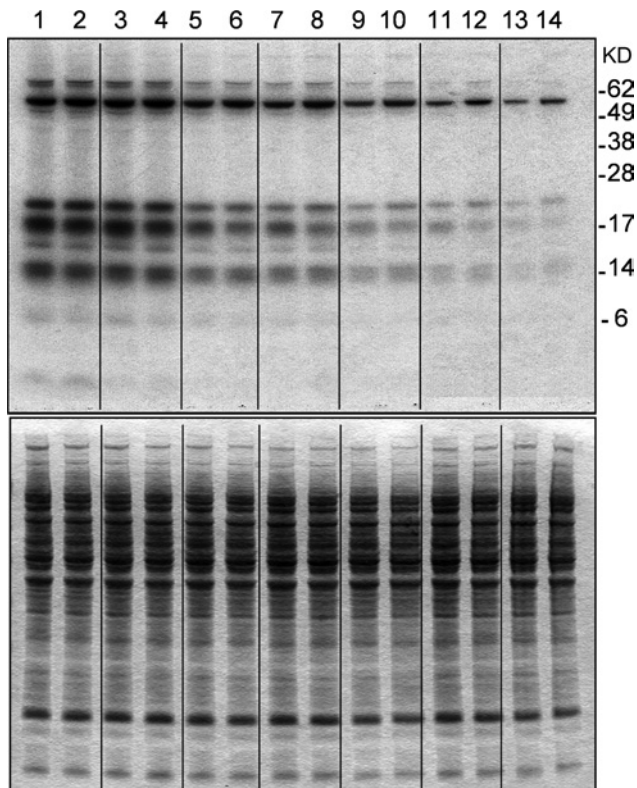


Figure 4 Loss of [⁷⁵Se]-labelling in selenoproteins in SPS1 knockdown cells

NIH3T3 cells were stably transfected with the siSPS1 construct (lanes 2, 4, 6, 8, 10, 12 and 14) or pU6Tet control construct (lanes 1, 3, 5, 7, 9, 11 and 13), grown in the presence of doxycycline for 3 days and then labelled overnight with [⁷⁵Se]. The cells were washed thoroughly to remove [⁷⁵Se], grown for 0 (lanes 1 and 2), 8 (lanes 3 and 4), 24 (lanes 5 and 6), 32 (lanes 7 and 8), 48 (lanes 9 and 10), 56 (lanes 11 and 12) and 72 h (lanes 13 and 14) and cell lysates electrophoresed on polyacrylamide gels as described in the Experimental section. Molecular masses are shown in kDa (KD) to the right-hand side of the upper panel. Coomassie Blue staining of the gel is shown in the lower panel as a protein loading control.

hierarchy in selenoprotein expression with some selenoproteins, such as GPx1, having low priority, and others, such as TR1, having high priority for selenium supply [19,20]. In the present study, supplementation of the media of control, SPS1 and SPS2 knockdown cells with selenite or selenocysteine revealed regulation of selenoprotein expression by both compounds. SPS1 knockdown cells manifested virtually the same response to medium supplementation with selenium as control cells, with the possible exception that TR1 may have been slightly over-expressed compared with the control cells. Furthermore, SPS1 did not appear to have a role in the turnover of selenoproteins, as the knockdown of this component did not affect selenoproteins over a 72 h period following the labelling of the cells with [⁷⁵Se].

The role of SPS2 is clearly defined as the catalyst for generating selenophosphate that is specifically used for selenoprotein synthesis (see the present study, [8] and references therein). What then is the role of SPS1? It belongs to the SPS protein family and is highly homologous to SPS2 (see [8] and references therein), but apparently has little or no effect on selenoprotein synthesis. A previous report indicated that mammalian SPS1 might have a role in recycling selenocysteine as tested in *E. coli* [15]. Our results, as shown in Figures 3 and 4, however, suggested that SPS1 is not involved in selenocysteine recycling in mammalian cells. SPS1 forms a complex with several proteins involved in

the biosynthesis of selenocysteine [21], which suggests that it has a role in some aspect of selenium metabolism. However, this role is clearly different from that previously suspected for this protein based on its homology to other members of the SPS family, and future studies will be required to establish its function in selenoprotein synthesis.

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