Selenophosphate synthetase genes from lung adenocarcinoma cells: *Sps1* for recycling L-selenocysteine and *Sps2* for selenite assimilation

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A labile selenium donor compound monoselenophosphate is synthesized from selenide and ATP by selenophosphate synthetase (SPS). In the present study, *Sps1* and *Sps2* were cloned from a cDNA library prepared from human lung adenocarcinoma cells (NCI-H441). The human lung *Sps1* has been cloned as an ORF of 1,179 bp, identical in sequence to that of the recently revised human liver *Sps1*. The in-frame TGA codon of the lung *Sps2* was genetically altered to TGT (Cys) to obtain the *Sps2Cys* gene. Expression of the recombinant plasmids containing *Sps1* or *Sps2Cys* was highly toxic to *Escherichia coli* host cells grown aerobically. Accordingly, the human lung *Sps* homologs were characterized by an *in vivo* complementation assay using a *selD* mutant strain. An added selenium source and a low salt concentration (0.1–0.25% NaCl) in the medium were required for reproducible and sensitive *in vivo* complementation. *Sps2Cys* effectively complemented the *selD* mutant, and the resulting formate dehydrogenase H activity was as high as that of WT *E. coli* MC4100. In contrast, only a weak complementation of the *selD* mutant by the *Sps1* gene was observed when cells were grown in selenite media. Better complementation with added L-selenocysteine suggested involvement of a selenocysteine lyase for mobilization of selenium. Based on this apparent substrate specificity of the *Sps1* and *Sps2* gene products we suggest that the *Sps1*-encoded enzyme depends on a selenium salvage system that recycles L-selenocysteine, whereas the *Sps2* enzyme can function with a selenite assimilation system.

In many biological systems, the concentration of sulfur-containing compounds is on the order of 1,000 times greater than their selenium analogs (1). Thus a selenium-specific pathway for biosynthesis of proteins that contain selenocysteine (SeCys) residues inserted as directed by the UGA codon is required. In *Escherichia coli* the insertion of selenium into selenium-dependent enzymes and Se-containing tRNAs requires the participation of the *selD* gene product (2). This protein, later identified as selenophosphate synthetase (SPS), forms a highly reactive, reduced selenium compound, monoselenophosphate (3). Monoselenophosphate is the product of the reaction catalyzed by SPS in which the γ-phosphoryl group of ATP is transferred to selenide and inorganic phosphate and AMP are formed (4, 5). The mechanism of this reaction has yet to be determined. The identification of an essential cysteine residue, Cys-17, in the N-terminal glycine-rich region of the *E. coli* enzyme (SELD) has led to the assumption that this residue behaves as a nucleophile in the hydrolysis of ATP (6, 7). Positional isotope exchange experiments demonstrated that an enzyme phosphoryl-intermediate is formed during catalysis (8). However, repeated attempts to trap an enzyme thiophosphate intermediate have failed, suggesting that Cys-17 of *E. coli* SELD may have a different catalytic role.

The *E. coli selD* gene product and its homologs present in mammals and *Drosophila* can be divided into two major groups. One group of SPS enzymes, which have a cysteine or SeCys residue at the site corresponding to Cys-17 in *E. coli* SELD, can catalyze the selenide-dependent formation of monoselenophosphate *in vitro* (3, 4, 9, 10). Replacement of Cys-17 with serine results in the complete loss of activity with ATP and selenide as substrates (6). Analysis of the total genomic sequences of *Methanococcus jannaschii* (11) and *Haemophilus influenzae* (12) revealed that the *selD* gene in these organisms possesses a TGA codon at the position of the *E. coli* Cys-17, indicating the presence of a SeCys residue (9, 10). Moreover, SeCys-containing variants also were identified in mouse and human enzymes (9). Replacement of the SeCys residue of the mouse enzyme with cysteine decreased but did not abolish enzyme activity (13), whereas changing Cys-17 of SELD to Ser destroyed catalytic activity (6). The second group of SELD homologs present in human (14), mouse (9), and *Drosophila* (15) share high sequence similarity with the bacterial SPS but lack a cysteine or SeCys residue in the position that corresponds to Cys-17 of the *E. coli* enzyme. The human homolog has a threonine substitution (14), and a homolog from *Drosophila melanogaster* contains an arginine (15). The overproduced threonine-containing human enzyme weakly complemented a *selD* lesion in *E. coli*, and transfection of the gene into mammalian cells resulted in an increased ^{75}Se labeling of mammalian selenium-dependent deiodinase (14).

Eukaryotic organisms including *D. melanogaster*, mouse, and human differ from bacteria and archaea in that they have a pair of *Sps* genes; one encodes a selenoenzyme capable of *in vitro* catalysis, and the other homolog encodes an enzyme exhibiting poor catalytic activity for selenide-dependent hydrolysis of ATP. Physiological roles of *Sps1* and *Sps2* remain to be elucidated, and the present study was initiated with an aim to clarify their roles in selenium homeostasis in mammals. It has been shown previously that *Sps2* is specifically expressed at very early stages of development *in vivo*, namely in mouse embryonic sites of hematopoiesis (9). Based on the demonstration that *Sps2* is a T cell activation gene, the up-regulation of *Sps2* in T cells may have a role in directing the production of selenophosphate to the synthesis of selenoproteins involved in the immune response rather than in processes related to cell growth and division.

We have cloned *Sps1* and *Sps2* genes from the human lung adenocarcinoma cell line NCI-H441, in which the selenoprotein thioredoxin reductase is synthesized in much higher amounts than in normal cells (16). The human lung adenocarcinoma *Sps1* was cloned as an ORF of 1,179 bp, and the nucleotide sequence was identical to that of the recently revised human liver *Sps1* sequence. *Sps*2 had two point mutations, one of which was a Thr301Ala mutation. The in-frame opal codon directing SeCys

Abbreviations: SPS, selenophosphate synthetase; SeCys, selenocysteine; FDH_H, formate dehydrogenase H; IPTG, β-D-thiogalactoside.

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in the lung *Sps2* was genetically altered to a Cys-encoding codon, and the resulting gene was designated *Sps2Cys*.

Materials and Methods

Materials. *E. coli* strains MC4100 and WL400 (Δ*selD*) (2) were obtained from the *E. coli* Genetic Stock Center at Yale University, New Haven, CT.

DNA Manipulation. General DNA manipulations were performed as described by Sambrook and Russel (17). Restriction enzymes and DNA polymerase were purchased from Toyobo (Osaka) and Takara (Kyoto). Plasmid DNA was isolated by using a GFX Micro Plasmid Prep Kit from Amersham Pharmacia Bioscience. DNA ligation was performed by using DNA ligation kit version 1 (Takara). Ultrafree-DA purchased from Millipore was used to recover DNA fragments from SeaKem (Rockland, ME) GTG agarose gels. DNA sequencing was performed by using a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia) and a model 310 capillary sequencer (Applied Biosystems). Each PCR was carried out in a 20-μl volume of 1× PCR buffer in the iCycler thermocycler system (BioRad).

Construction of Recombinant Plasmid Containing the *Sps2Cys* Gene. NCI-H441 cells were purchased from the American Type Culture Collection and maintained in RPMI medium 1640 with 10% heat-inactivated FBS (Sigma) at 37°C in humidified air with 5% CO_2. When cells reached about 70% confluence mRNA was isolated by using the poly(A)pure mRNA isolation kit (Ambion, Austin, TX). cDNA synthesis was performed by using 3 μg of mRNA and an Omniscript RT kit (Qiagen, Valencia, CA). Human lung *Sps2* gene was amplified by using a HotStarTaq PCR kit (Qiagen) and a set of primers (Sps2Up, 5′-CGGGATCCATGGCGGAAGCCTCGGCGA-3′; and Sps2Dw, 5′-CCCAAGCTTTCACGAGCTAGGCTCAGAGGA-3′. The underlined sequences indicate *Bam*HI and *Hin*dIII sites, respectively. PCR products were ligated into the pCR2.1 vector by using a TOPO TA cloning kit (Invitrogen). Recombinant plasmids were subjected to DNA cycle sequencing in the sense and antisense directions. The in-frame TGA codon of human lung *Sps2* was changed to the cysteine-encoding codon TGT with a QuikChange Site-Directed Mutagenesis Kit (Stratagene) using a set of primers (*Sps2Cys*Up, 5′-ATGAAGGGCTGTGGCTGCAAG-3′ and *Sps2Cys*Dw, 5′-CTTGCAGCCACAGCCCTTCAT-3′) and pCR-*Sps2* as a template. The resulting gene *Sps2Cys* was subcloned into the *Bam*HI and *Hin*dIII sites of pQE30 (Qiagen), and the recombinant plasmid was designated pQE30*Sps2Cys*.

Construction of Recombinant Plasmid Containing *Sps1* Genes. The *Sps1* gene was amplified from the cDNA library of human lung adenocarcinoma cells by using a HotStarTaq PCR kit (Qiagen) and a pair of primers (Sps1UP, 5′-CGCGGATCCATGTCTACGCGGGAGTCCTTTA-3′ and Sps1Dw, 5′-ACGCGTCGACTCACGTTTTGAGTGGCCACTTG-3′. The underlined sequences indicate *Bam*HI and *Sal*I sites, respectively. The primers were designed based on the *Sps1* sequence of 1,152 bp reported for human liver cDNA (14). The PCR product was subjected to DNA sequencing, and the *Sps1* gene was found to have a frame-shift mutation caused by C insertion at 1130, resulting in extension of the ORF to 1,179 bp. To confirm the *Sps1* sequence, another cDNA library was prepared by using a ReverTraAce RT-PCR kit (Toyobo), and the gene was amplified by using a KOD-plus DNA polymerase kit (Toyobo) with a set of primers (Sps1Up2, 5′-CGCGGATCCATGTCTACGCGGGAGTCCTTTAACC-3′ and Sps1Dw2, 5′-ACGCGTCGACTTAAGAGGTGGCCCCGGGTGT-3′. The underlined sequences indicate *Bam*HI and *Sal*I sites, respectively. PCR products were incubated with 2 mM dATP and 10 units of Ex-*Taq*DNA polymerase (Takara) at 70°C for 3′ adenylation and cloned in the pCR2.1 vector. The DNA sequencing in the sense and antisense directions confirmed the C1130 insertion. The C1130 of human lung *Sps1* was removed by QuikChange site-directed mutagenesis using a set of primers (ΔC1130Up, 5′-ATCGAGGTCGCACACAAGTGGCCACT-3′ and ΔC1130Dw, 5′-AGTGGCCACTTGTGTGCGACCTCGAT-3′) and pCR-*Sps1* as a template. The entire subcloned gene was sequenced to confirm that no other mutation were present. Recombinant genes *Sps*1 and *Sps1*Δ*C1130* were subcloned in *Bam*HI and *Sal*I sites of the expression vector pQE30, and the expression plasmids were designated pQE30*Sps1* and pQE30*Sps1*Δ*C1130*.

Multiple Alignment and Phylogenetic Tree. The multiple protein sequence alignment was carried out with CLUSTAL W (18) using the sequences of *Methanopyrus kandleri selD* (GenBank accession no. AE010430), *M. jannaschii selD* (GenBank accession no. F64498), *Geobacter sulfurreducens selD* (GenBank accession no. AAR33938), *D. melanogaster Sps*1 (GenBank accession nos. O18373, O18597, and Q9V700) and *Sps*2 (GenBank accession no. Q8IPC0), *E. coli selD* (GenBank accession nos. P16456 and P78172), *H. influenzae selD* (GenBank accession no. P43911), *Yersinia pestis selD* (GenBank accession no. Q8ZEK1), *Mus musculus* (mouse) *Sps1* (GenBank accession no. Q8BH69) and *Sps2* (GenBank accession no. P97364), *Caenorhabditis elegans* probable SPS (GenBank accession no. O62461), *Caenorhabditis briggsae* hypothetical SPS (GenBank accession no. CAE58592), *Campylobacter jejuni selD* (GenBank accession no. Q9PMF9), *Thermoanaerobacter tengcongensis selD* (GenBank accession no. Q8R8W3), *Salmonella typhimurium selD* (GenBank accession no. NP460263), *Aquifex aeolicus selD* (GenBank accession no. O67139), *Rhizobium meliloti selD* (GenBank accession no. Q931D0), and *Eubacterium acidaminophilum selD1* (GenBank accession no. AJ245960) and *selD2* (GenBank accession no. AJ249161). The phylogenetic tree was calculated by the neighbor-joining method applied to the distance of difference derived from the multiple alignments (18).

Plasmid Stability Test. A colony from the transformation plate was inoculated into 2 ml of LB medium containing 50 μg/ml carbenicillin and incubated until the culture became slightly turbid. Cells were streaked on agar plates containing carbenicillin, and single colonies were used to inoculate 2 ml of LB-1% glucose containing either 0 or 50 μg/ml carbenicillin. After growth at 37°C slightly turbid cultures were diluted to 10^{-5}, and cells were streaked on LB agar containing 1% isopropyl β-D-thiogalactoside (IPTG) with or without 50 μg/ml added ampicillin; 10^{-6} dilutions of cells were plated on LB agar with or without added ampicillin.

Complementation of an *E. coli selD* Mutation by Human Lung *Sps1* and *Sps2Cys*. In this study, *E. coli*, strain WL400 (Δ*selD*) cells (2) transformed with the *Sps1* or *Sps2Cys* gene, were tested for formate dehydrogenase H (FDH_H) activity in an *in vitro* assay (19). Fresh transformant cells were streaked on LB-glucose agar plates and grown for 2 days at 30°C in Anaerocult A mini filled with CO_2 (Merck). The plates then were overlaid with soft agar containing 1 mg/ml benzyl viologen, 0.25 M sodium formate, and 25 mM KH_2PO_4 (19–21). Development of a purple color upon reduction of the viologen dye indicated that the bacterial cells contained catalytically active FDH_H.

Results

RT-PCR of *Sps1* and *Sps2* from Human Lung Adenocarcinoma Cells. Human *Sps1* and *Sps2* genes were amplified from the cDNA library synthesized from human lung adenocarcinoma cells (NCI-H441) (Fig. 1). The PCR product of *Sps*1 gave an intense signal on agarose gel electrophoresis. In contrast, a very weak

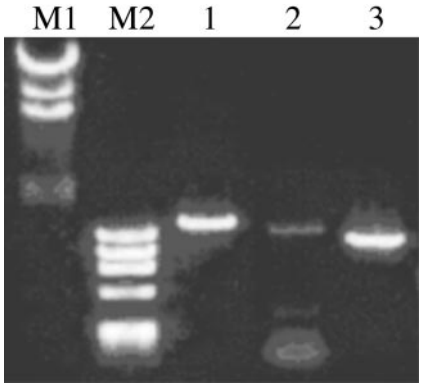

Fig. 1. RT-PCR of human lung *Sps* genes from mRNA extracted from NCI-H441. Lane M1, λ/*Hin*dIII molecular markers; lane M2, ΦX174/*Hae*III molecular markers; lane 1, cytosolic thioredoxin reductase (TrxR1) amplified as a positive control; lane 2, human lung *Sps2* gene amplified in the presence of Q solution; lane 3, *Sps1* gene amplified in the presence of 1.5 mM MgCl2 without Q solution.

amplification signal was obtained for *Sps2*, whose specific amplification required the presence of Q solution (Qiagen); no signals were obtained without the optimizer solution even when Mg concentration and thermal programs were varied.

Construction of an Expression Plasmid Containing the *Sps2Cys* Gene. The PCR product for *Sps2* was cloned into the TA cloning vector pCR2.1, and the resulting plasmid was designated pCR*Sps2*. DNA sequence analysis revealed two point mutations in the ORF of 1,347 bp; A901 → G resulted in a Thr301Ala change but G1221A was a silent mutation of Val-407. A mutant cDNA containing a TGT (Cys) codon in place of TGA (SeCys) was obtained by oligonucleotide mutagenesis, and the resulting plasmid was designated pCR-*Sps2Cys*. pCR-*Sps2Cys* was digested with *Bam*HI and *Hin*dIII, and the 1.2-kb fragment was inserted into the corresponding site of pQE30 to yield pQE30*Sps2Cys*.

Construction of Expression Plasmids Containing *Sps1*. The *Sps1* gene was cloned from a human lung cDNA library prepared by using a Omniscript RT-PCR kit and a HotStarTaq PCR kit. The PCR primer was designed on the sequence of human liver *Sps1* (14), and nucleotide sequencing identified a frame-shift mutation caused by the C1130 insertion. Another cDNA library was made with a ReverTraAce RT-PCR kit, and the *Sps1* gene was amplified by using KOD-plus DNA polymerase. The frame shift caused by the C1130 insertion was reproduced in the second round of RT-PCR, and the human lung *Sps1* gene encoding an ORF of 1,179 bp was designated *Sps1*. The human liver *Sps1* sequence, which recently was revised as an ORF encoding 1,179 bp, now was completely consistent with the sequence of the human lung *Sps1*.

Multiple Alignment and Phylogenic Tree. The Cys-17 in *E. coli* SPS (SELD) occurs in a glycine-rich sequence, -Gly-16–Cys-17–Gly-18–Cys-19–Lys-20–Ile-21–Ser-22–Pro-23. Multiple alignments of amino acid sequences of bacterial, archaea, and eukaryote SPS variants identified conserved sequences corresponding to the *E. coli* glycine-rich sequence (Figs. 2 and 3). The residue corresponding to Gly-16 in *E. coli* SPS is conserved in all of the sequences. Residues corresponding to Gly-18 and Cys-19 also are found in many SPS homologs, including human lung SPS1 and SPS2, but they are replaced with alanine in bacterial and archea SPS variants. Lys-20 is a catalytically essential residue in *E. coli* SELD (22), and it is conserved in all of the SPS homologs.

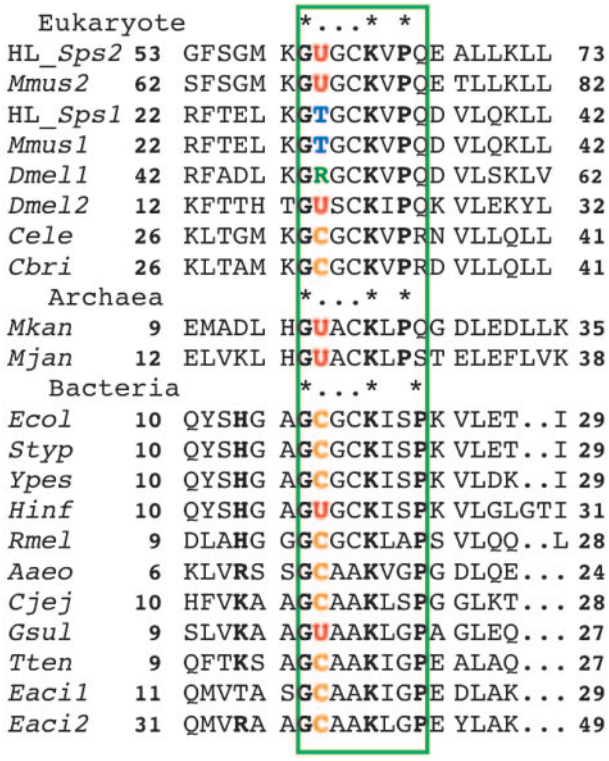

Fig. 2. Partial amino acid sequence alignment of SPS homologs from the human lung cancer cell (HuLung), mouse (*Mmus*), *D. melanogaster* (*Dmel*), *C. elegans* (*Cele*), *C. briggsae* (*Cbri*), *Methanopyrus kandleri* (*Mkan*), *M. jannaschii* (*Mjan*), *E. coli* (*Ecol*), *S. typhimurium* (*Styp*), *Y. pestis* (*Ypes*), *H. influenzae* (*Hinf*), *R. meliloti* (*Rmel*), *A. aeolicus* (*Aaeo*), *C. jejuni* (*Cjej*), *G. sulfurreducens* (*Gsul*), *T. tengcongensis* (*Tten*), and *E. acidaminophilum* (*Eaci*1 and *Eaci*2).

One of three hydrophobic residues, valine, isoleucine, or leucine, follows the Lys-20, and a helix-breaking L-proline occurs either in position 22 or 23. If the highly conserved region from Gly-16 to Ile/Val/Leu-21 is in either an α-helix or a loop conformation, Lys-20 could be close enough to interact with Cys-17, thus effectively lowering the pK_a value for the thiol ionization.

Expression of Recombinant *Sps* Genes in *E. coli*. Despite extensive efforts, expression of recombinant human lung *Sps* genes in *E. coli* cells has not been successful. We have subcloned *Sps* genes in a number of vectors, including pUC18, pKK233-3, pET32, and pQE30, and used host strains JM109, DH5α, BL21(DE3), and M15. *E. coli* ABLE-K strain (Toyobo) that suppresses the plasmid copy number by 1/10th that of normal *E. coli* hosts also was tested with the recombinant plasmids, pQE30*Sps1* and pQE30*Sps2Cys*. However, no Coomassie blue-stained bands corresponding to SPS proteins were detected on SDS/PAGE. Expression of *Sps1* and *Sps2Cys* genes in *E. coli* WL400 cells under anaerobic conditions gave similar results.

Plasmid Stability Test. Recombinant plasmids normally are stable and are retained by a very high fraction of host cells even after growth for many generations in the absence of antibiotics. However, problems of plasmid instability can arise when a gene whose product is toxic to the host cell is present in the plasmid. To determine the fraction of cells carrying the recombinant *Sps* gene, freshly transformed cells grown in the absence or presence of 50 μg/ml carbenicillin were plated on four agar media of differing composition (Table 1). When *E. coli* WL400 cells harboring the pQE30 vector were grown under aeration, most of

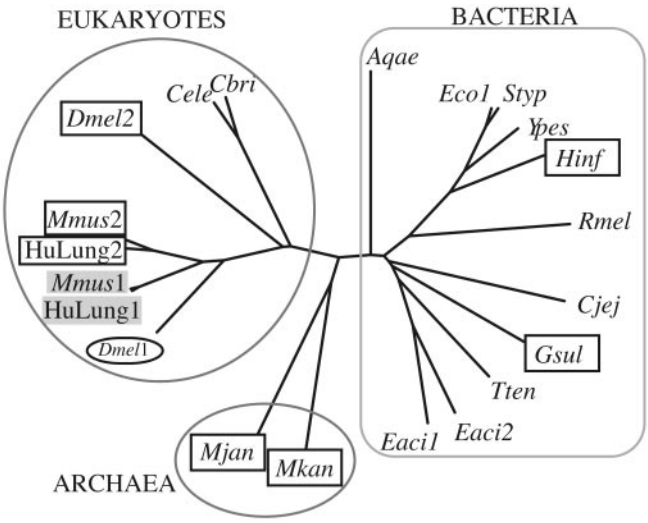

Fig. 3. Dendrogram showing the divergence of SPS homologues. Boxes around human lung *Sps2* (HuLung2), mouse *Sps2* (Mmus2), *D. melanogaster Sps2* (*Dmel2*), *H. influenzae selD* (Hinf), *G. sulfurreducens Sps* (*Gsul*), *M. jannaschii Sps* (*Mjan*), and *Methanopyrus kandleri Sps* (*Mkan*) denote their special character as selenoenzymes. Mouse *Sps1* (*Mmus1*) and human lung *Sps1* (HuLung1) designated in shaded boxes appeared to have Thr, whereas the *D. melanogaster Sps1* (*Dmel1*) has Arg residue at the corresponding position, suggested by the multiple sequence alignment of all of the available SPS homologs.

the cells retained the plasmid and grew on the ampicillin-containing agar. When the host cell transformed with the *Sps2Cys* gene was grown in the absence of carbenicillin, the recombinant plasmid was very unstable and none of the cells retained the recombinant plasmid. When the pQE30*Sps2Cys* transformants were grown in the presence of carbenicillin, there were fewer viable cells and the plasmid was barely retained in the surviving cells. The β-lactamase enzyme, secreted in the medium in the early stages of growth, may have degraded the selective antibiotic, thus allowing growth of cells lacking the plasmid. The human lung *Sps1* gene appeared to be stably retained in the *E. coli* host cells, but growth was much slower than the cells with pQE30 vector alone. Normally, addition of IPTG to the agar will prevent colony formation by cells containing both the inducible gene and a functional target plasmid, but not the growth of cells lacking the plasmid or mutants that have lost the ability to express target DNA. The large fraction of cells able to grow on IPTG-containing LB agar suggested that a majority of the recombinant cells were unable to express the recombinant gene. When the growth test was carried out in the presence of carbenicillin, most of the surviving cells were found to be insensitive to IPTG induction. *Sps1*Δ1130C was made from the *Sps1* gene by removing the C1130. The behavior of recombinant *Sps1*Δ1130C gene resembled that of *Sps2*, indicating the recombinant plasmid was unstable in the host cell. The cells transformed with *Sps1*Δ1130C gene could not retain the recombinant plasmid either in the absence or presence of carbenicillin.

The Medium Optimization for *in Vivo* Complementation Assay. In previous studies, the *in vivo selD* complementation tests have been carried out by monitoring FDH_H activity of *E. coli* cells grown in media such as LB agar containing D-glucose (20, 21). We found that lower NaCl concentrations (0.1–0.25%) in the media and supplementation with selenium markedly improved the reproducibility and sensitivity of the complementation assay. Accordingly, the agar medium used consisted of 1.6% polypepton, 1% yeast extract, 1% agar, and 0.1% NaCl (pH 7), 10 μM Na_2MoO_4, and 1 μM Na_2SeO_3 or 1 μM L-SeCys. Fresh transformant cells were streaked on agar plates and incubated at 30°C in a plastic bag filled with CO_2. Addition of IPTG to the agar medium interfered with color development and was not included. Sodium selenite, up to 10 μM or L-SeCys, 0.1 or 1 μM, served as a satisfactory selenium source. However, 10 μM L-SeCys inhibited the growth of *E. coli* MC4100 (WT), WL400 (Δ*SelD*), and *E. coli* WL400 cells transformed with *Sps2Cys* or *Sps1*. Selenite, >10 μM, also is toxic for many bacterial species (23).

Complementation of *selD* Lesion by Human Lung *Sps1* and *Sps2Cys*. The human lung *Sps1* or *Sps2Cys* genes cloned in pQE30 complemented the bacterial *selD* deficiency to different extents. Although the active site SeCys-60 was genetically replaced with a Cys residue, human lung *Sps2Cys* allowed *E. coli* WL400 cells to produce FDH_H activity on the agar media supplemented with 1 μM sodium selenite. The FDH_H activity detected for WL400/pQE30*Sps2Cys* was as high as that of WT MC4100 in which intact *selD* was functional. When selenium was supplied as 1 μM L-SeCys, the detected FDH_H activity was less than with sodium selenite, suggesting that selenite was a better selenium source for *Sps2Cys* than L-SeCys (Fig. 4*A*). In contrast, *E. coli* cells transformed with the *Sps1* gene showed low FDH_H activity when sodium selenite was added as a selenium source. Interestingly, the *Sps1* gene allowed *E. coli* host cells to produce detectable FDH_H activity when L-SeCys was added as the selenium source (Fig. 4*B*). Although the method of detection of *E. coli* FDH_H activity is indirect and thus a nonquantitative determination of *in vivo* SPS activity, the higher FDH_H activity observed suggests that the human lung SPS1 generated monoselenophosphate from selenium that was mobilized from free L-SeCys. This finding is consistent with previous reports on the mobilization of selenium from L-SeCys, providing a better selenium source for *SelD in vivo*.

Table I. Plasmid stability test

		Without carbenicillin*			With carbenicillin*		
Media	pQE30	*Sps2Cys*	*Sps1*	*Sps1Δ1130C*	*Sps2Cys*	*Sps1*	*Sps1Δ1130C*
LB agar	2,600	1,390	220	450	70	70	860
+ Ampicillin	2,300	0	220	0	0	170	0
+ IPTG	290	745	68	202	35	165	45
+ Ampicillin, IPTG	300	0	6	12	8	79	0

All viable cells can grow on the plate with no additives; cells that retain plasmid will grow on the plates containing ampicillin; cells that have lost plasmid or lost the ability to express target DNA will grow in the presence of IPTG; and mutants that retain plasmid but have lost the ability to express target DNA will grow in the presence of both ampicillin and IPTG.

**E. coli* WL400 was transformed with pQE30*Sps2Cys,* pQE30*Sps1,* and *Sps1Δ1130C* genes, respectively, and grown in 2 ml of LB containing 1% glucose with 50 g/ml carbenicillin or without carbenicillin.

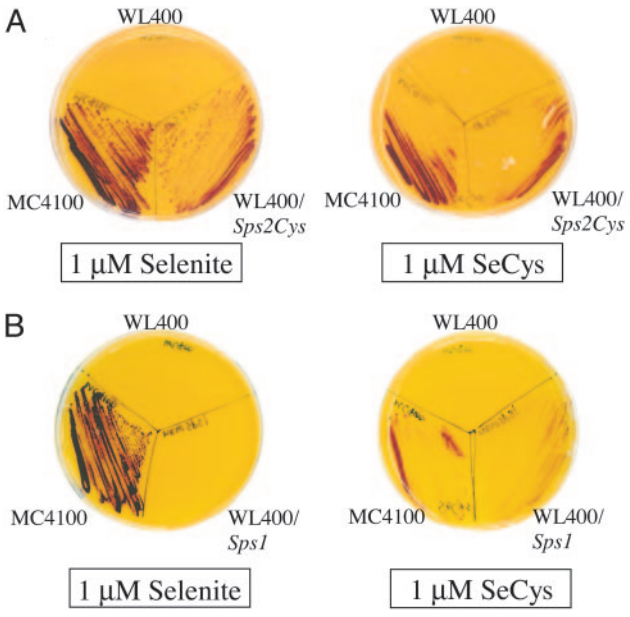

Fig. 4. *SelD* complementation by human lung *Sps* genes. *E. coli* MC4100 (WT), WL400 (*selD*), and WL400 transformed with pQE30*Sps2Cys* (*A*) or pQE30*Sps1* (*B*) were grown anaerobically at 30°C on a plate containing 10 μM sodium molybdate and 1 μM sodium selenite or 1 μM L-SeCys. Soft agar containing 0.75% agar, 1 mg/ml benzyl viologen, 0.25 M sodium formate, and 25 mM KH_2PO_4 was laid over the culture. Colonies with active FDH_H reduced benzyl viologen, which develops a deep blue color.

Discussion

The present study has demonstrated that human lung adenocarcinoma cells (NCI-H441) possess mature mRNA molecules encoding *Sps1* and *Sps2.* Recombinant genes, *Sps1* and *Sps2Cys*, were highly toxic to *E. coli* host cells when grown under aerobic conditions. However, the two gene products could be characterized in an *in vivo* complementation assay using the *selD*-deficient *E. coli* WL400 strain grown under anaerobic conditions. For optimal expression of the *Sps* genes it was necessary to decrease the NaCl content of the usual rich medium and add Se and Mo supplements. The mutant *Sps2Cys* complemented the *selD* mutation as effectively as the intact *selD* does in WT strain MC4100. The presence of *Sps2Cys* allowed the *E. coli* host to produce the SeCys-containing FDH_H when the medium was supplemented with sodium selenite or L-SeCys. In contrast, complementation with *Sps1* was relatively ineffective, particularly when selenite was used as a selenium source for FDH_H synthesis.

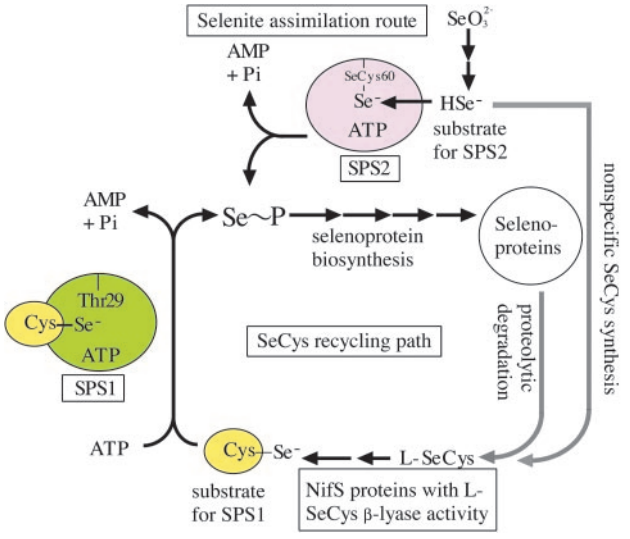

Fig. 5. Hypothetical selenium assimilation routes in the lung adenocarcinoma cell NCI-H441. Up-regulation of SPS2, capable of using selenide derived from selenite, provides a bypass route, which directly converts selenide into monoselenophosphate, leading to an increased cellular selenium pool. The SeCys-60 residue in *Sps2* is proposed to provide a selenide binding site for enzyme-substrate complex formation. SPS1 that lacks a SeCys or Cys residue in the corresponding glycine-rich sequence would require a selenium-delivery system in which activated selenium is supplied as a perselenide (-S-SeH) derivative.

The specific gene amplification of *Sps2* from the cDNA library of lung adenocarcinoma cells required the addition of Q solution; yet the yield was still smaller than the amplified *Sps1* gene when compared on agarose gel electrophoresis. Because the two homologs share high sequence homology, it is likely that the *in vivo* transcription of *Sps2* was much lower than *Sps1* in the adenocarcinoma cells.

It was reported by Kim *et al.* (13) that replacement of SeCys by cysteine in fetal mouse *Sps2* reduced but did not eliminate enzyme activity. Accordingly, the human lung adenocarcinoma *Sps2* was genetically altered in this study to the cysteine-containing homolog. The *selD*-defective host harboring recombinant *Sps2Cys* gene exhibited FDH_H activity equal to that of the WT *E. coli* strain, indicating a high catalytic potency of *Sps2Cys* gene product for selenophosphate synthesis. In fact, human lung *Sps2Cys* was able to use both selenite-derived and L-SeCys-derived selenium as substrate. In contrast, *Sps1*, the human homolog containing threonine, only weakly complemented a *selD* mutation in *E. coli* when sodium selenite was the selenium source. The human lung *Sps1* specifically required SeCys-derived selenium for monoselenophosphate production, suggesting the involvement of L-SeCys β-lyase enzymes. A similar requirement was observed with a mutant form of SPS from *E. coli* that contained serine in place of Cys-17. This mutant was inactive *in vitro* with selenide as selenium source, but when selenium mobilized from SeCys by a lyase protein was supplied a low, but detectable, level of selenophosphate was produced (20). Based on these observations it seems that human *Sps1* and the *E. coli* Cys-17/Ser SPS mutant are functional analogs in terms of selenium substrate requirement.

The apparent participation of L-SeCys β-lyase enzymes in selenophosphate biosynthesis in bacteria was first observed for selenoprotein A of the glycine reductase complex in *Clostridium sticklandii*. ^{75}Se derived from [^{75}Se]-SeCys was incorporated into the selenoprotein more efficiently than $^{75}SeO_3^{2-}$ (24). It also was demonstrated that the NifS protein from *Azobactor vinelandii* (25) and the NifS-like proteins from *E. coli* (20) effectively mobilized selenium from free SeCys and the selenium was used for monoselenophosphate production by SPS. Furthermore, Lacourciere (23) demonstrated that selenium mobilized from L-SeCys by NifS-like protein was specifically incorporated into *E. coli* FDH_H.

NifS-like proteins have a conserved cysteine residue near the active site, and the residue is proposed to bind selenium and deliver it as a substrate for selenium metabolizing enzymes such as SPS (26). It seems likely that the human lung *Sps1* also used the *E. coli* system that provides protein-bound selenium as the substrate.

The substrate specificity of human lung *Sps* gene products characterized in the present study has allowed us to hypothesize that *Sps2* functions in the pathway of *de novo* synthesis of selenophosphate from selenite after reduction of the latter by intracellular thiols. The reduced selenium is bound to the SeCys-60 residue of *Sps2* to form an enzyme-substrate complex (Fig. 5). Alternatively, for *Sps1* catalysis, NifS-like proteins in

mammalian cells would supply an elemental form of selenium derived via a SeCys-salvage pathway. In bacteria selenide can be converted to a trace amount of free L-SeCys through nonspecific incorporation via the cysteine biosynthetic pathway. Alternatively, SeCys can be formed from selenoproteins through proteolysis during protein turnover. These pathways could provide a source of L-SeCys for the NifS-like proteins. The lethal effect of recombinant *Sps* genes on the *E. coli* host under aerobic conditions presumably could be caused by the high levels of this reactive selenium compound produced in the cells. If the enzyme reaction product is equally toxic for mammalian cells, the cellular damage may be controlled by limiting the supply of required substrate for *Sps1* product formation or by extremely low expression of *Sps2*, respectively.

Further study is required to identify the mammalian selenium-delivery protein that can serve as a substrate for the Thr-containing SPS1 protein and also to experimentally prove that a SeCys/Cys residue in SPS2 protein/*E. coli* SELD actually functions as a substrate-binding residue for the reduced form of selenite.

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