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Evidence for Direct Roles of Two Additional Factors, SECp43 and Soluble Liver Antigen, in the Selenoprotein Synthesis Machinery*

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Selenocysteine (Sec) is inserted into selenoproteins co-translationally with the help of various cis- and trans-acting factors. The specific mechanisms of Sec biosynthesis and insertion into protein in eukaryotic cells, however, are not known. Two proteins, SECp43 and the soluble liver antigen (SLA), were previously reported to interact with tRNA$^{[Ser]Sec}$, but their functions remained elusive. Herein, we report that knockdown of SECp43 in NIH3T3 or TCMK-1 cells using RNA interference technology resulted in a reduction in the level of methylation at the 2′-hydroxylribosyl moiety in the wobble position (Um34) of Sec tRNA$^{[Ser]Sec}$, and consequently reduced glutathione peroxidase 1 expression. Double knockdown of SECp43 and SLA resulted in decreased selenoprotein expression. SECp43 formed a complex with Sec tRNA$^{[Ser]Sec}$ and SLA, and the targeted removal of one of these proteins affected the binding of the other to Sec tRNA$^{[Ser]Sec}$. SECp43 was located primarily in the nucleus, whereas SLA was found in the cytoplasm. Co-transfection of both proteins resulted in the nuclear translocation of SLA suggesting that SECp43 may also promote shuttling of SLA and Sec tRNA$^{[Ser]Sec}$ between different cellular compartments. Taken together, these data establish the role of SECp43 and SLA in selenoprotein biosynthesis through interaction with tRNA$^{[Ser]Sec}$ in a multiprotein complex. The data also reveal a role of SECp43 in regulation of selenoprotein expression by affecting the synthesis of Um34 on tRNA$^{[Ser]Sec}$ and the intracellular location of SLA.

Several factors required for the specific incorporation of selenocysteine (Sec)[2] as the 21st amino acid in the genetic code have been identified, and their roles have been assessed in mammalian cells and tissues (see Refs. 1 and 2 for reviews). For example, the tRNA that specifically incorporates Sec into protein in response to Sec UGA codons has been characterized wherein the amino acid is biosynthesized on the tRNA following its aminoacylation with serine by seryl-tRNA synthetase (2). Thus, Sec tRNA has been designated Sec tRNA$^{[Ser]Sec}$. There are two species of this tRNA in mammalian cells that differ by a single methyl group on the ribosyl moiety at position 34 (designated Um34; Ref. 3). The form that does not contain Um34 is 5-methoxycarbonylmethyluridine (mcm^{5}U) and that containing Um34 is 5-methoxycarbonylmethyl-2′-*O*-methyluridine (mcm^{5}Um). The two species appear to have different roles in protein synthesis, wherein mcm^{5}Um is associated with the synthesis of stress-related selenoproteins (*e.g.* glutathione peroxidase 1 [GPx1]), and mcm^{5}U is associated with the synthesis of housekeeping selenoproteins (*e.g.* thioredoxin reductases) (4, 5).

A specialized stem-loop structure in the 3′-untranslated region of mammalian selenoprotein genes, designated the SEC insertion sequence (SECIS) element, was identified that accounted for UGA codon specificity in dictating Sec incorporation into protein (6). The SECIS elements that have been identified in the 24 known selenoproteins in rodents and 25 known selenoproteins in humans (7) fall into two classes that differ in the occurrence of a ministem that is adjacent to the apical loop of the structure (8). A SECIS-binding protein, designated SBP2, was purified from various mammalian sources including liver, testes, and hepatoma cells (9) and shown to be required along with the SECIS element in selenoprotein mRNA for Sec insertion into protein (10). Recently, a ribosomal protein, L30, was found to bind SECIS elements and to have a role in mediating Sec incorporation at the ribosomal level (11). A specific elongation factor for Sec-tRNA$^{[Ser]Sec}$ has also been characterized (12, 13), and it recognizes only the selenocysteinyl-tRNA$^{[Ser]Sec}$ form of the tRNA and not, for example, the seryl-tRNA$^{[Ser]Sec}$ form (13). Additional factors that have a role in the biosynthesis of Sec and its insertion into protein in mammals are selenophosphate synthetases 1 (SPS1; Ref. 14) and 2 (SPS2) (15). These enzymes apparently are responsible for generating an active form of selenium for its utilization in Sec synthesis and/or possibly other selenium-requiring reactions. Interestingly, SPS2 is a selenoprotein and thus has been implicated in the autoregulation of selenoprotein synthesis (15).

There are two other factors that also have been shown to be associated with Sec tRNA$^{[Ser]Sec}$, SECp43 (16) and the soluble liver antigen, SLA (17). Protein overlay analysis provided evidence for the interaction of SECp43 with a 48-kDa protein in HeLa cytoplasmic extracts (16), whereas SLA was detected in patients with an autoimmune chronic hepatitis as an autoantibody that precipitated Sec tRNA$^{[Ser]Sec}$ from human cell extracts and recognized a 48-kDa protein (17). However, the identity of the 48-kDa protein, the requirements of the SECp43 complex formation, and the roles of either SECp43 or SLA were not determined.

Herein, we investigated the role of SECp43 and SLA using RNAi knockdown technology (18–21). The targeted knockdown of SECp43

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[2] The abbreviations used are: Sec, selenocysteine; SECIS, SEC insertion sequence; SLA, soluble liver antigen; RNAi, RNA interference; GFP, green fluorescent protein; BFP, blue fluorescent protein; CHO, Chinese hamster ovary; siRNA, small interfering RNA; nt, nucleotide; MSLA, mouse SLA; HSLA, human SLA.

resulted in the reduced formation of Um34 on Sec tRNA$^{[Ser]Sec}$ and affected selenoprotein expression in a similar manner as mice lacking or containing reduced levels of mcm^{5}Um (4, 5, 22). These observations suggest that SECp43 has a role in Um34 synthesis. To target the knockdown of SLA, we generated a construct that contained five knockdown target sites in SLA mRNA, which resulted in dramatically reduced levels of this mRNA in transfected cells. Interestingly, transfection of cells with a construct encoding both SECp43 and SLA knockdown sequences had a much more pronounced effect on selenoprotein biosynthesis than either individual knockdown construct suggesting that, although both components likely have separate roles in selenoprotein synthesis, they also act in concert. In addition, we found that SLA forms a complex with SECp43 and Sec tRNA$^{[Ser]Sec}$ and found that SECp43 was located primarily in the nucleus, whereas SLA was in the cytoplasm. However, co-transfection of both protein genes resulted in the nuclear translocation of the SLA gene product suggesting that the roles of these two components are interdependent.

EXPERIMENTAL PROCEDURES

Materials—^{75}Selenium (specific activity 1000 Ci/mmol) was purchased from the Research Reactor Facility, University of Missouri, Columbia, MO, [α-^{32}P]dCTP (specific activity ~6000 Ci/mmol) was from NEN Corporation, and [^{3}H]serine (specific activity 29 Ci/mmol) and Hybond Nylon N$^+$ membranes were from Amersham. NuPage 10% polyacrylamide gels, polyvinylidene difluoride membranes, Superscript II reverse transcriptase, cell culture fetal bovine serum, Dulbecco's modified Eagles medium, α-minimum essential medium, Lipofectamine 2000, TRIzol reagent, and Hygromycin B were purchased from Invitrogen. DNA polymerase I large Klenow fragment was obtained from New England BioLabs, pSilencer 2.0 U6 Hygro vector was from Ambion, the pTriEx-4 Hygro vector was from Novagen, and SuperSignal West Dura extended duration substrate was from Pierce. Anti-His-tag antibodies were purchased from Qiagen, horseradish peroxidase-conjugated secondary antibodies were from Sigma, and SLA antibodies, generated against the peptide, TFRGFMSHTNNYPC (amino acids 358–371 of human SLA), were prepared by Spring Valley Laboratories, Inc. (Woodbine, MD). pEGFP-N1 and pEGFP-C3 vectors, green fluorescent protein (GFP) and blue fluorescent protein (BFP) were purchased from Clontech. A Storm Image system (Molecular Dynamics) was used to scan Phosphor screens. All other chemicals and reagents were obtained commercially and were commercial products of the highest grade available.

Mammalian Cell Lines, Cell Culture, and Transfection—Mouse kidney (TCMK-1) and embryonic fibroblast (NIH3T3) cells were obtained from ATCC. Chinese hamster ovary AA8 (CHO) cells encoding 10 copies of the wild type mouse Sec tRNA$^{[Ser]Sec}$ transgene were obtained as given (22). NIH3T3 and TCMK-1 cells were cultured in Dulbecco's modified Eagle's medium, and CHO cells were cultured in α-minimum essential medium, and both media were supplemented with 10% fetal bovine serum. Transfections were carried out using Lipofectamine 2000 according to the manufacturer's instructions. Media for cells grown in the presence of selenium were supplemented with 300 nM sodium selenite.

Generation of SECp43 Expression and SECp43 and SLA siRNA Constructs—The SECp43 clone and antibodies to SECp43 were obtained as given (16). The SECp43 clone was amplified using PCR and cloned into the pTriEx-4 Hygro vector for SECp43 expression. The sequence was confirmed by DNA sequencing. The pU6 vector used for expressing siRNA constructs was constructed using the pSilencer 2.1-U6 Hygro vector as the backbone. Changes were inserted into the vector as follows: 1) GC (positions 468–469) were changed to A making the U6 promoter more homologous to the corresponding wild type gene, 2) the EcoRI site was deleted at position 4110, and 3) XhoI and MfeI sites were added at positions 384 and 393, respectively. The latter two changes made it possible to insert multiple siRNA target sequences into the vector. The modified vector was designated as pU6-m4 Hygro.

For knocking down SECp43 gene expression, five separate 19-nt sequences, 5′-TTATCCGAAACCGCCTCAC-3, 5′-GCCGAGAAGTGTTTGCATA-3′, 5′-TTGTGAAATTCACAGATGA-3′, 5′-GCAGTACCAGAACTACTAT-3′, and 5′-CCATGCAGACATATGAAGA-3′, were selected from within mouse SECp43 cDNA as being unique to this gene using the online service "si*DESIGN*" of Dharmacon Research, Inc. Oligonucleotides containing 19 nt of sense and 19 nt of complementary (antisense) sequences with an intervening AGAGAACTT loop were synthesized and inserted into the pU6-m4 Hygro vector according to the manufacture's instructions. The sequences of all five of SECp43 siRNA constructs were verified by sequencing. The five constructs and the negative control plasmids were separately transfected into NIH3T3 and TCMK-1 cells. After stabilizing the transfected cells with 0.8 mg/ml hygromycin, several colonies were selected from plates, and the SECp43 mRNA levels were determined by northern hybridization as described below. Based on mRNA levels, the best siRNA target sequence was identified as 5′-GCAGTACCAGAACTACTAT-3′, and its siRNA construct was used in subsequent experiments.

For knocking down mouse SLA (designated MSLA) gene expression, five separate 19–24-nt sequences, 1) 5′-GCTTGCAGTCATGGATAGCAACAA-3, 2) 5′-GAAATATCACCGGATCGTCCAA-3′, 3) 5′-GCTCATCACCTTCCAATACGT-3′, 4) 5′-GTCAACAACGCTTATGGATTA-3′, and 5) 5′-GAACAGACTAGAGCAAGTG-3′, were selected from within MSLA cDNA as being unique to this gene using the online service si*DESIGN* of Dharmacon Research, Inc. Oligonucleotides containing between 19 and 24 nt of sense and 19 to 24 nt of complementary (antisense) sequences with an intervening AGAGAACUU loop were synthesized and inserted separately within the BamHI and HindIII cloning sites of the pU6-m4 Hygro vector. The sequences of all five of MSLA siRNA constructs were determined for confirmation. For combining the five siRNA transcription units, two of the constructs (encoding the second and fourth siRNA sequences shown above) were digested with EcoRI and XhoI to release the U6-siRNA units. Two additional constructs (encoding the first and third siRNA sequences shown above) were digested with MfeI and XhoI. The released second and fourth U6-siRNA unit fragments were purified and inserted separately into the digested first and third siRNA vectors. Then the first and the second U6-siRNA units were combined in tandem into one vector, whereas the third and the fourth U6-siRNA units were combined in tandem in another vector. Repeating the same procedure, all of the five U6-siRNA units were inserted into the same vector. The combined MSLA construct and the negative control plasmids were transfected into NIH3T3 and TCMK-1 cells. After stabilizing the transfected cells with 0.8 mg/ml hygromycin, MSLA mRNA levels were determined by northern blot hybridization as described below.

To generate a double knockdown vector encoding the siRNA targeting site for SECp43 mRNA and the five targeting sites for MSLA, the MSLA siRNA construct was digested with EcoRI and XhoI to release the five MSLAU6-siRNA units as a single fragment. The multiple MSLAU6-siRNA fragment was then inserted into the SECp43 siRNA construct, which was initially digested with MfeI and XhoI.

Labeling of Selenoproteins—Cultured cells were washed three times in phosphate-buffered saline to remove exogenous selenium, suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 25

μCi/ml of ^{75}Se added to the medium, and grown an additional 24 h before harvesting as described (4, 22). Cells were again washed in phosphate-buffered saline, suspended in 1× SDS loading buffer, and electrophoresed on NuPage 10% polyacrylamide gels along with standard molecular weight markers to assess labeled selenoprotein sizes. The developed gels were stained with Coomassie Blue, dried, and exposed to Phosphor screens. The screens were then scanned using a Storm Image (PhosphorImager) system (4, 22). Band densities of GPx1 were quantified using ImageQuant Version 5.3 (Molecular Dynamics).

Northern Blot Analyses—Total RNA was isolated from various transfected cells using TRIzol reagent. Equal amounts (15 μg) from the different cells were loaded onto the gels, and the developed gels were transblotted onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled probe and analyzed with a PhosphorImager for northern blot analysis as given (4, 22). The GPx1 probe was prepared and used as described (4, 22). The SECp43, MSLA, and glyceraldehyde-3-phosphate dehydrogenase probes were generated by either PCR or reverse transcription-PCR using Superscript II reverse transcriptase and Universal Reference RNA (4, 22). The sizes of all reverse transcription-PCR-generated probes were in the 500–1200-bp range. To detect an array of mRNAs on the same membrane, membranes, after an initial probing for SECp43 mRNA, were stripped in boiling buffer (0.1× SSC and 0.1% SDS), hybridized with a second probe, analyzed, stripped, hybridized with another probe, and analyzed, etc., using successively labeled MSLA, GPx1, and glyceraldehyde-3-phosphate dehydrogenase cDNA probes. Hybridized membranes were exposed to Phosphor screens, and the screens were then scanned using a Strom Image System (Molecular Dynamics). The density of GPx1 northern blotting bands were quantified using ImageQuant Version 5.2 (Molecular Dynamics).

Immunoprecipitation, Western Blot, and Sec tRNA$^{[Ser]Sec}$ Northern Blot Analyses—CHO or NIH3T3 cells (1×10^7) were harvested and suspended in 1 ml of lysis buffer (1× TBS with 0.2% Triton X-100). Either 50 μl of anti-His-agarose or 5 μl of preimmune sera, anti-SLA sera, or anti-SECp43 antibody along with 50 μl of protein A/G-agarose were added to 500 μg of lysis proteins in 1 ml of lysis buffer. After binding at 4 °C overnight, the agarose beads were washed three times with 1 ml of 1× TBS with 0.1% Tween 20 and suspended in 30 μl of 1× protein loading buffer (for Western blotting) or 1× TBE/urea loading buffer (for northern blotting). 10 μl were loaded onto gels for Western blotting or for Sec tRNA$^{[Ser]Sec}$ northern hybridization.

Proteins obtained by immunoprecipitation (see above) or from 50 μg of proteins from SECp43 siRNA, MSLA siRNA, or SECp43/MSLA siRNA-transfected cell lysate and control cell lysate were electrophoresed on NuPage 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes containing proteins obtained by immunoprecipitation were then incubated with either mouse anti-His (1:2000 dilution), or rabbit-anti-human-SLA (1:250), whereas those containing cell lysates were incubated with rabbit-anti-mouse GPx1 (1:1000). Horseradish peroxidase-conjugated secondary antibodies (1:30000) were then applied, and the membranes were washed with 0.1% TBS-T, incubated in SuperSignal West Dura Extended Duration Substrate, and exposed to x-ray film as given (4, 22). Sec tRNA$^{[Ser]Sec}$ obtained by immunoprecipitation (see above) was electrophoresed on 15% TBE/urea polyacrylamide gels and transferred to Hybond nylon membranes, and the membranes were hybridized with ^{32}P-labeled Sec tRNA$^{[Ser]Sec}$ probe and analyzed with a PhosphorImager as above.

Isolation, Aminoacylation, and Chromatography of tRNA—SECp43 siRNA-transfected and control-transfected NIH3T3 cells were grown in 75-cm^2 plates either with 300 nM SeO_3^{2-} or without selenium. After reaching 80–90% confluence, the cells were collected, and total tRNA was isolated from ~1 g of cells as described (23). Transfer RNA was aminoacylated with [^{3}H]serine in the presence of 19 unlabeled amino acids and rabbit reticulocyte aminoacyl-tRNA synthetases (24), and the resulting aminoacyl-tRNAs were fractionated on a RPC-5 column (25) as described (4, 22, 26).

Constructs for Subcellular Localization and Fluorescence Confocal Microscopy—GFP expression constructs were prepared using pEGFP-N1 and pEGFP-C3 vectors, respectively. The coding region of mouse SECp43 was PCR-amplified from NIH3T3 cDNA with forward primer 5′-CCT*AAGCTT*ATGGCGGCCAGCCTATGG-3′ and reverse primer 5′-GCT*GGATCC*ATCATAGCTGGGATCTCTG-3′ and cloned into the HindIII/BamHI sites of pEGFP-N1. To generate SLA-GFP expression construct, the human sequence (accession number NM 153825) was PCR-amplified from HEK-293 cDNA with forward primer 5′-CCT*GAATTC*ATGGACAGCAACAATTTC-3′ and reverse primer 5′-TCG*GTCGAC*CCTGAAGAAGCATCCTGGTATGTG-3′ (underlined, italicized sequences specific endonuclease restriction sites) and cloned into the EcoRI/SalI sites of pEGFP-N1. The human sequence (designated HSLA) was chosen because it shares 86.9% identity with its predicted mouse homolog (27). In addition, C-terminal GFP fusion proteins were generated, correspondingly, using vector pEGFP-C3 for comparison. To study the co-localization of SECp43 and HSLA, the GFP sequence of SECp43-GFP was cut with BamHI/HpaI and replaced with BFP to generate a SECp43-BFP fusion protein. For fluorescence confocal microscopy, images were collected using an Olympus FluoView 500 laser scanning microscope (Melville, NY).

RESULTS

Development of Multi-siRNA Constructs for Efficient Knockdown of SECp43 and MSLA mRNA Expression—Because SECp43 (16) and MSLA (17) were previously implicated in binding Sec tRNA$^{[Ser]Sec}$, we explored the possibility of whether these two proteins function in the same pathway, or possibly in concert, by knocking down their gene expression using RNAi technology. Constructs of the five 19-nt sequences selected for targeting knockdown of mouse SECp43 (see "Experimental Procedures") were examined for their ability to reduce the mRNA level of this protein in NIH3T3 cells. The construct corresponding to nucleotides 594–612 within the coding sequence was the most effective of those examined and was sufficient for targeting the removal of SECp43 mRNA (comparative data not shown). This siRNA construct, which is designated SECp43 siRNA construct, was used in all subsequent experiments.

When five distinct MSLA siRNA targets were assayed individually for their knockdown potential, they were unsuccessful in reducing mRNA levels more than 50–60% (data not shown). Therefore, we generated a construct that encoded all five of the RNAi sequences unique to the corresponding cDNA as described under "Experimental Procedures."

Finally, we devised a strategy to knockdown both proteins in the same cell line. The SECp43 siRNA target sequence was cloned into the multi-siRNA vector-construct developed in targeting MSLA knockdown such that the new construct contained the five MSLA targeting sequences and the SECp43 targeting sequence. This construct was designated SECp43/MSLA siRNA construct.

SECp43 and MSLA siRNAs Target Their Respective mRNAs for Removal—Northern blot analysis was used to document the effect of the SECp43 and MSLA siRNA on inhibiting the expression of the corresponding target mRNAs following transfection of NIH3T3 and TCMK-1 cells with the appropriate construct. TCMK-1 cells were transfected with either the SECp43 siRNA construct or the MSLA siRNA construct (Fig. 1*A*), and NIH3T3 cells were transfected with

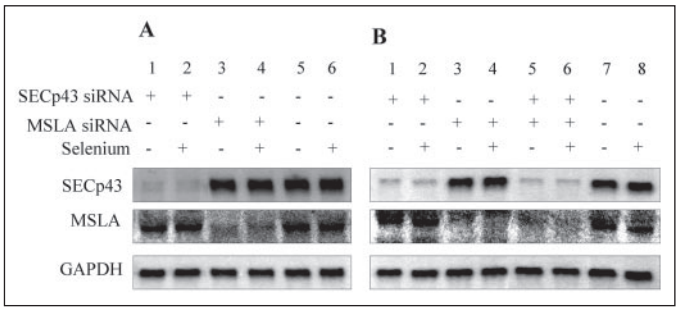

FIGURE 1. **Assessment of SECp43 and MSLA mRNA levels in the corresponding siRNA knockdown cells by northern blotting.** *A*, TCMK-1 cells were transfected with the SECp43 siRNA construct (*lanes 1* and *2*), the MSLA siRNA construct (*lanes 3* and *4*), or the control vector (*lanes 5* and *6*) and grown in the presence (*lanes 2*, *4*, and *6*) or absence of supplemental selenium (*lanes 1*, *3*, and *5*). *B*, NIH3T3 cells were transfected with the SECp43 siRNA construct (*lanes 1* and *2*), the MSLA siRNA construct (*lanes 3* and *4*), the SECp43/MSLA siRNA construct (*lanes 5* and *6*), or the control vector (*lanes 7* and *8*) and grown in the presence (*lanes 2*, *4*, *6*, and *8*) or absence of supplemental selenium (*lanes 1*, *3*, *5*, and *7*). Total RNA was extracted for northern blotting and hybridized with the appropriate ^{32}P-labeled cDNA probes as described under "Experimental Procedures." Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) RNA was used as a control to monitor sample loading.

either of these siRNA constructs or the SECp43/MSLA siRNA construct (Fig. 1*B*). Because selenoproteins and Sec $tRNA^{[Ser]Sec}_{mcm5Um}$ are responsive to selenium status (2, 4) and because the two protein factors were implicated in selenoprotein metabolism (16, 17), the effects of the siRNA constructs on the targeted mRNAs were examined in cells grown in the presence or absence of supplemental selenium. SECp43 siRNA effectively knocked down its mRNA in both NIH3T3 and TCMK-1 cells when the cells were grown either in the absence (compare *lanes 1* and *5* in Fig. 1*A* with *lanes 1* and *7* in Fig. 1*B*, respectively) or the presence of supplemental selenium (compare *lanes 2* and *6* in Fig. 1*A* with *lanes 2* and *8* in Fig. 1*B*, respectively). MSLA RNAi also inhibited the expression of its mRNA in NIH3T3 and TCMK-1 cells transfected with the multi-siRNA construct grown in the absence (compare *lanes 3* and *5* in Fig. 1*A* with *lanes 3* and *7* in Fig. 1*B*, respectively) or presence of supplemental selenium (compare *lanes 4* and *6* in Fig. 1*A* with *lanes 4* and *8* in Fig. 1*B*, respectively). As expected, the SECp43/MSLA siRNA was also effective in knocking down both targeted mRNAs in NIH3T3 cells, independent of selenium status (compare *lanes 5* and *7* with *6* and *8* in Fig. 1*B*).

SECp43 and MSLA siRNA Effects on Selenoprotein Expression, ^{75}Se-labeling—A simple means of monitoring selenoprotein synthesis is to label the respective treated and untreated cells with ^{75}Se and resolve the labeled proteins by gel electrophoresis. To examine the effect of the siRNA knockdown constructs on selenoprotein expression, NIH3T3 cells were stably transfected with the SECp43 siRNA, MSLA siRNA, and SECp43/MSLA siRNA constructs or the control vector, and the cells were grown in the presence and absence of supplemental selenium and labeled with ^{75}Se (Fig. 2). The *upper panel* in Fig. 2*A* shows the labeling of selenoproteins, whereas the *lower panel* shows the Coomassie Blue-stained gel from the transfected cells following gel electrophoresis. Clearly, the double knockdown SECp43/MSLA siRNA construct had a greater effect on selenoprotein synthesis than either of the individual siRNA knockdown constructs (compare *lanes 5* and *6* (double knockdown construct) with *lanes 1–4* (SECp43 and MSLA siRNA constructs, respectively) and *lanes 7* and *8* (control construct)). MSLA siRNA did not appear to affect selenoprotein synthesis (compare *lanes 3* and *4* with *lanes 7* and *8*), but SECp43 siRNA appeared to selectively reduce the amount of GPx1 when the cells were grown in the presence of supplemental selenium (compare *lanes 2* and *8*). The relative intensities of the GPx1 bands were quantified to examine more closely the effect of SECp43 knockdown on GPx1 expression (Fig. 2*B*). The level of GPx1 in

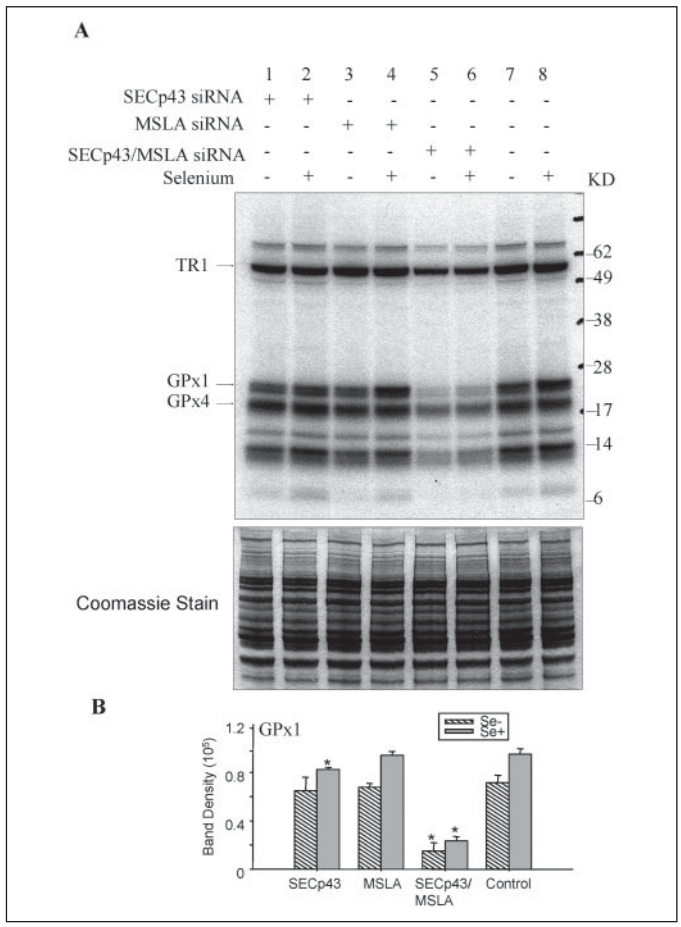

FIGURE 2. **^{75}Se-Labeling of SECp43 siRNA knockdown cells.** NIH3T3 cells were transfected with the SECp43 siRNA construct (*lanes 1* and *2*), the MSLA siRNA construct (*lanes 3* and *4*), the SECp43/MSLA siRNA construct (*lanes 5* and *6*), or the control vector (*lanes 7* and *8*), grown in the presence (*lanes 2*, *4*, *6*, and *8*) or absence of supplemental selenium (*lanes 1*, *3*, *5*, and *7*), and then labeled overnight with ^{75}Se. Cell lysates were run on gels, and the gels were stained with Coomassie Blue(*lower panel*), dried, and exposed to a PhosphorImager screen (*upper panel*) as described under "Experimental Procedures." The average densities of GPx1 bands from three separate labelings are shown in *B*. The asterisk (*) indicates a *p* value equal to or lower than 0.01.

cells transfected with the SECp43 siRNA construct was significantly reduced in cells grown in the presence of selenium (*p* value was 0.01, see legend to Fig. 2).

SECp43 siRNA Effects on GPx1 Expression, Northern and Western Blot Analyses—To further assess the effect of SECp43 siRNA on GPx1 expression, NIH3T3 cells transfected with either the SECp43 siRNA construct or control vector were examined for GPx1 mRNA expression by northern blot analysis (Fig. 3*A*) and for GPx1 expression by Western blot analysis (Fig. 3*C*). In addition, we examined the effects of MSLA and SECp43/MSLA siRNA on the expression of GPx1 mRNA and the protein itself. SECp43 siRNA reduced GPx1 mRNA expression in both TCMK-1 and NIH3T3 cells when the cells were grown in the presence or absence of selenium (Fig. 3*A*, compare *lanes 2* and *8* with *lanes 1* and *7*, respectively), whereas MSLA siRNA did not reduce GPx1 mRNA expression (see *lanes 3*, *4*, *7*, and *8*). The double knockdown construct, SECp43/MSLA siRNA, had a dramatic effect on GPx1 mRNA expression in NIH3T3 cells, especially in the presence of selenium (Fig. 3*A*, compare *lanes 6* and *8*). To better illustrate the effects of the SECp43 siRNA and the double knockdown constructs on GPx1 mRNA and GPx1 expressions, the relative amounts of these components in cells transfected with the different constructs were quantified (Fig. 3*B*). The most pronounced effects on GPx1 mRNA and protein reductions

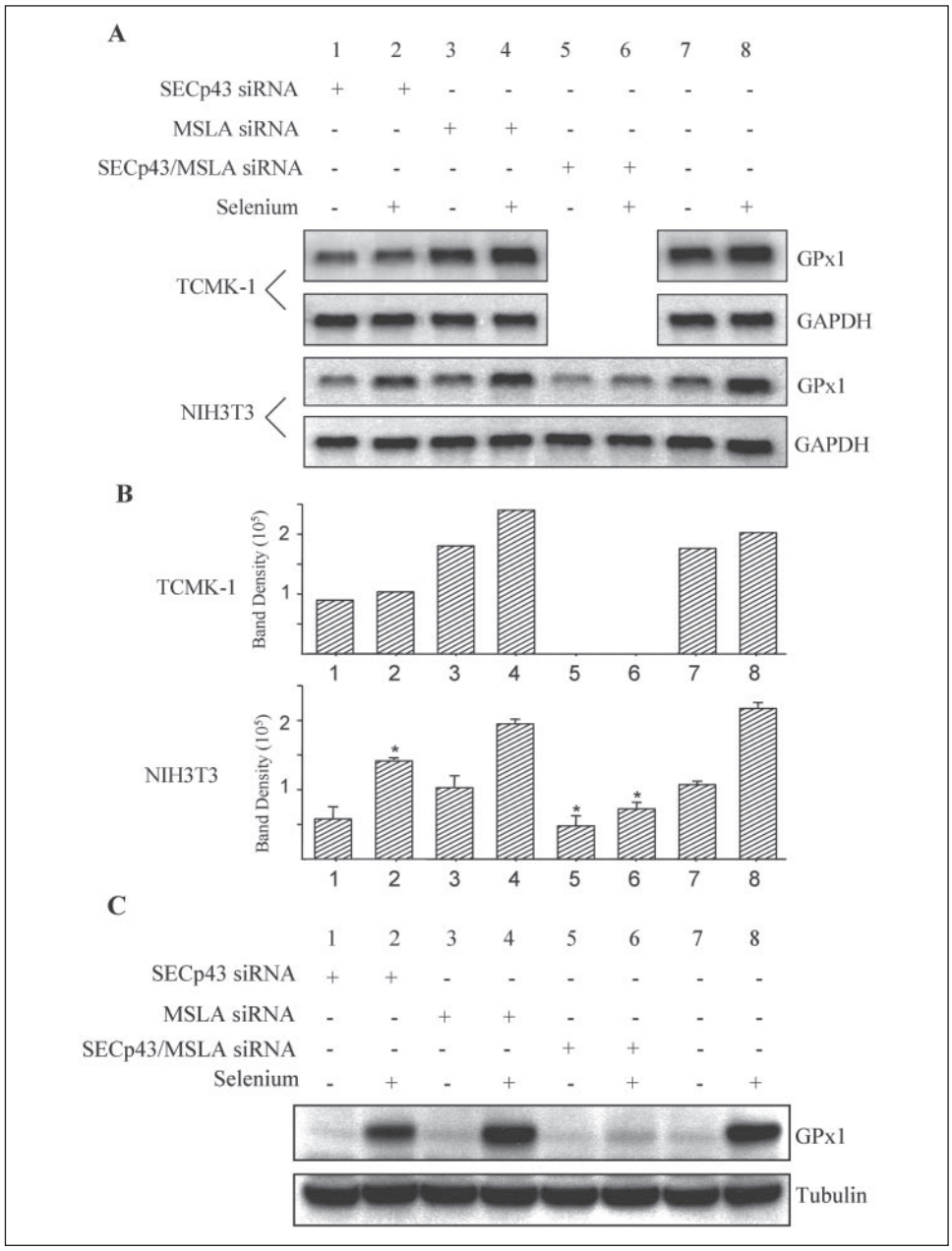

FIGURE 3. **Assessment of GPx1 mRNA and protein levels by northern and Western blotting in extracts of SECp43, MSLA, and SECp43/MSLA siRNA knockdown cells.** *A*, in the *top two rectangular boxes*, TCMK-1 cells were transfected with the SECp43 siRNA construct (*lanes 1* and *2*), the MSLA siRNA construct (*lanes 3* and *4*) or with the control vector (*lanes 7* and *8*), and the cells were grown in the presence (*lanes 2*, *4*, and *8*) or absence (*lanes 1*, *3*, and *7*) of supplemental selenium. TCMK-1 cells were not transfected with the SECp43/MSLA siRNA construct. NIH3T3 cells were transfected with the SECp43 siRNA construct (*lanes 1* and *2*), the MSLA siRNA construct (*lanes 3* and *4*), the SECp43/MSLA siRNA construct (*lanes 5* and 6), or the control vector (*lanes 7* and *8*) and grown in the presence (*lanes 2*, *4*, *6*, and *8*) or absence of supplemental selenium (*lanes 1*, *3*, *5*, and *7*), and extracts were prepared for northern (*A*, *lower two long rectangular boxes*) or Western blotting (*C*) as described under "Experimental Procedures." Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used in northern blots in *A*, and tubulin was used in *B* as the controls to monitor sample loading. The densities of GPx1 bands from northern blotting of TCMK-1 cell RNA are shown in the *upper panel* of *B*, and the average densities of GPx1 bands from three separate northern blottings of NIH3T3 cell RNA are shown in the *lower panel* of *B*. The asterisk (*) indicates a *p* value equal to or lower than 0.01 in each case with the exception of that in *lane 5*, which is 0.03.

occurred in the presence of the double knockdown construct. In addition, the SECp43 siRNA construct caused a significant reduction in GPx1 mRNA that was more apparent in the TCMK-1 than NIH3T3 cells.

As expected, Western blot analysis of GPx1 in extracts of the various siRNA knockdown cells (Fig. 3*C*) yielded similar results as that observed for GPx1 mRNA. It should also be noted that GPx1 mRNA is thought to be subject to nonsense-mediated decay in cells and tissues that are deficient in selenium, which also lack or have lower levels of Sec $tRNA^{[Ser]Sec}_{mcm5Um}$ (see Refs. 4 and 5 and references therein). Therefore, the reduced amounts of GPx1 mRNA observed by northern analysis and GPx1 observed by Western analysis in cells grown in the absence of selenium (Fig. 3) were anticipated.

SECp43 siRNA Effects on Um34 Synthesis—Because NIH3T3 cells had reduced levels of GPx1 in SECp43 siRNA-transfected cells than control cells (see above), and this effect is also a characteristic of mammalian cells and tissues containing lower amounts of the mcm^5Um isoform (4, 5), we examined the Sec $tRNA^{[Ser]Sec}$ population in NIH3T3 cells transfected with SECp43 siRNA or the control vector. Total tRNA was isolated from both transfected cells lines that were grown in the presence and absence of supplemental selenium and the tRNA was aminoacylated with [^{3}H]serine. The resulting [^{3}H]seryl-tRNA was chromatographed on a RPC-5 column to separate [^{3}H]seryl-$tRNA^{[Ser]Sec}_{mcm5U}$ and [^{3}H]seryl-$tRNA^{[Ser]Sec}_{mcm5Um}$ (Fig. 4, *A* and *B*). The tRNA species lacking Um34 (mcm^5U) eluted earlier from the column than the form containing Um34 (mcm^5Um) (4, 22). Comparison of the distributions of the two species in transfected cell lines grown in the presence of supplemented selenium showed that there was more mcm^5U than mcm^5Um in the cells containing SECp43 siRNA, whereas the reverse was evident in cells transfected with the control vector (Fig. 4, *A1* and *B1*, respectively). As the distribution of the two Sec $tRNA^{[Ser]Sec}$ species is known to be responsive to selenium status (2, 4, 5, 22), the proportion of mcm^5Um was reduced substantially in the control cell line, as expected, when grown in the absence of supplemental selenium as compared with

that grown in the presence of added selenium (compare Fig. 4, *B1* and *B2* and see Refs. 2, 4, and 22). The level of mcm^{5}Um was even more reduced in cells containing the SECp43 siRNA construct and grown in the absence of supplemental selenium (compare *A2* to *A1* and to *B2*) further demonstrating the effect of SECp43 on Um34 synthesis.

Cellular Interrelationship of SECp43 and SLA, Co-precipitation—To examine the cellular interrelationship of SECp43 and SLA, CHO cells were transfected with the SECp43 expression construct encoding a His-tag at the C terminus of the protein, and the resulting cell extracts were treated with SLA or His-tag antibodies (Fig. 5*A*). Extracts from CHO cells transfected with the SECp43 expression construct that were immunoprecipitated with anti-His-tag (to bring down SECp43), yielded a strong signal on Western blots using antibodies against SLA (see Fig. 5*A*, *lane 2*). Extracts from CHO cells transfected with the control protein expression construct encoding a His-tag at its C terminus did not respond on Western blots (Fig. 5*A*, *lane 1*). Immunoprecipitation with anti-SLA antibodies further confirmed the presence of the *in vivo* SECp43-MSLA complex (Fig. 5*A*, *lane 4*). Interestingly, similar results were obtained on northern blots by assaying for the attachment of Sec tRNA$^{[Ser]Sec}$ to the SECp43-SLA complex in NIH3T3 cell extracts transfected with either the SECp43 siRNA or MSLA siRNA construct or the control vector (Fig. 5*B*). Immunoprecipitation of SECp43 from extracts of NIH3T3 cells transfected with either the SECp43 or MSLA siRNA constructs pulled down very little Sec tRNA$^{[Ser]Sec}$ compared with that observed with the control (compare *lanes 1* and *2* with *lane 3* in the *left panel* of Fig. 5*B*). Immunoprecipitation of MSLA from extracts of NIH3T3 cells transfected with either the SECp43 or MSLA siRNA constructs manifested reduced pull down of Sec tRNA$^{[Ser]Sec}$ compared with that observed with the control (compare *lanes 4* and *5* with *lane 6* in *right panel* of Fig. 5*B*). However, less Sec tRNA$^{[Ser]Sec}$ pull down occurred with the MSLA knockdown cells compared with SECp43 knockdown cells (Fig. 5*B*, compare *lanes 4* and *6* with *lanes 5* and *6*, respectively). Clearly, the targeted removal of either one of these factors affected the binding of Sec tRNA$^{[Ser]Sec}$ to the complex, although tRNA$^{[Ser]Sec}$ binding appears to be far more stringently dependent on SLA.

Cellular Interrelationship of SECp43 and SLA, Subcellular Localization—To investigate the cellular distribution of SECp43, both N- and C-terminal GFP fusion constructs were generated. SECp43-GFP was predominantly present in the nucleus of NIH3T3 cells 48 h after transfection (Fig. 6). Because N- or C-terminal GFP fusion might affect localization, a GFP-SECp43 construct was also tested that confirmed the subcellular localization of SECp43 as being in the nucleus (data not shown).

We also investigated the cellular location of SLA and used the corresponding HSLA as the model protein. For this purpose, HSLA·GFP fusion constructs were generated. Transfection experiments revealed the cytosolic accumulation of SLA·GFP, whereas GFP expression alone resulted in the homogenous nuclear and cytosolic distribution (Fig. 6).

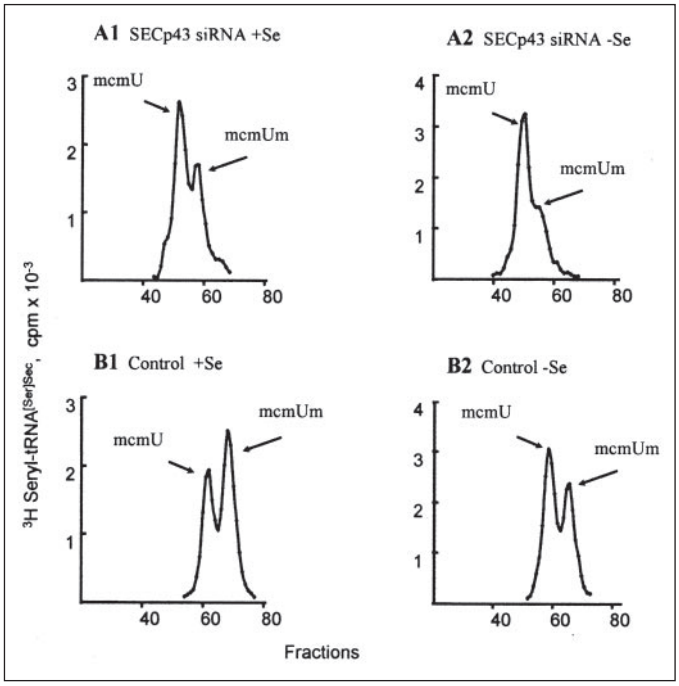

FIGURE 4. **Effect of SECp43 siRNA on Sec tRNA$^{[Ser]Sec}$ Um34 synthesis.** Total tRNA was isolated from NIH3T3 cells and aminoacylated with [^{3}H]serine, and the resulting [^{3}H]seryl-tRNA was chromatographed on a RPC-5 column as given under "Experimental Procedures". [^{3}H]Seryl-tRNA$^{[Ser]Sec}$ from cells were transfected with the SECp43 siRNA construct (*A panels*) or with the control vector (*B panels*) and grown in the presence (designated *+Se*) or absence of supplemental selenium (designated *−Se*) as shown. Variations in elution positions of the two tRNA$^{[Ser]Sec}$ isoforms shown in the figure are a reflection of the use of slightly different NaCl gradients in the different runs.

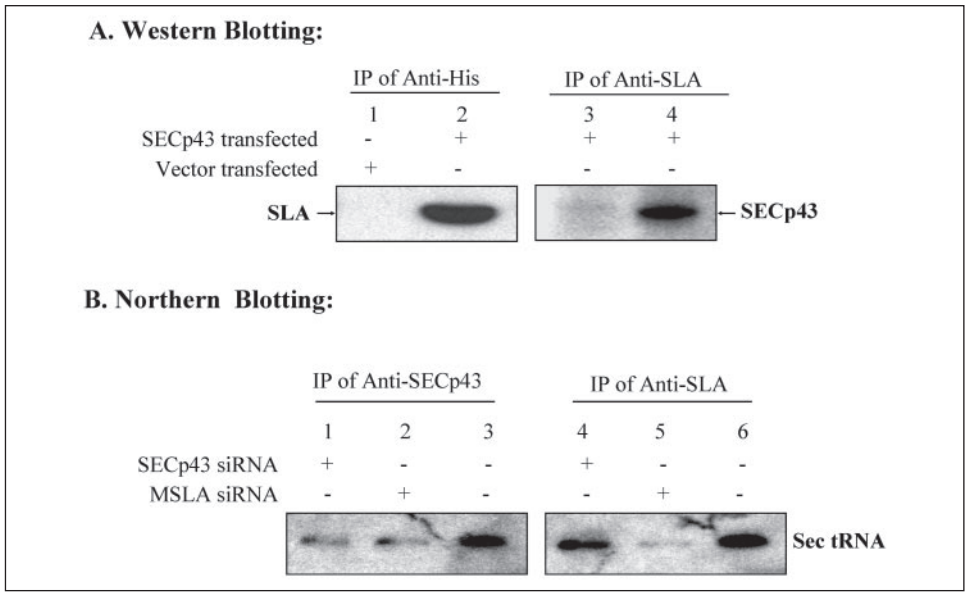

FIGURE 5. **Immunoprecipitation (*IP*) of SECp43 and MSLA.** *A*, lysates of CHO cells transfected with the SECp43 expression construct (*lanes 2–4*) or the vector encoding the His-tag control protein (*lane 1*) were mixed with anti-His antibody (*lanes 1* and *2*), anti-SLA sera (*lane 4*), or preimmune sera (*lane 3*). The mixture was run on gels, the gels were transblotted onto membranes, and Western blots were prepared as described under "Experimental Procedures." *B*, lysates of extracts of NIH3T3 cells transfected with the SECp43 siRNA or MSLA siRNA constructs or the control vector were mixed with either anti-SECp43 antibody (*lanes 1–3*) or anti-SLA sera (*lanes 4–6*). The mixture was run on gels, the gels were transblotted, and northern blots were prepared by hybridizing with ^{32}P-labeled Sec tRNA$^{[Ser]Sec}$ cDNA as described under "Experimental Procedures."

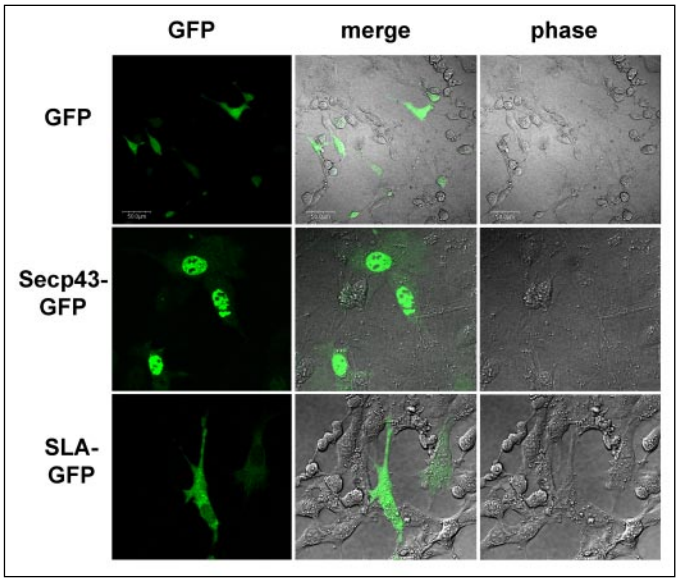

FIGURE 6. **Localization of SECp43 and HSLA.** Confocal images of NIH3T3 cells expressing GFP-fused SECp43, HSLA, and GFP control protein at 48 h posttransfection after a single transfection are illustrated as follows. A set of three images is shown for each construct. *Left panels*, green fluorescence corresponding to transiently expressed fusion proteins; *center panels*, fluorescence of the fusion proteins merged with the phase contrast; and *right panels*, images obtained by using phase contrast. GFP-transfected cells were photographed at 20× magnification, and SECp43-GFP- and HSLA-GFP-transfected cells were photographed at 40× magnification.

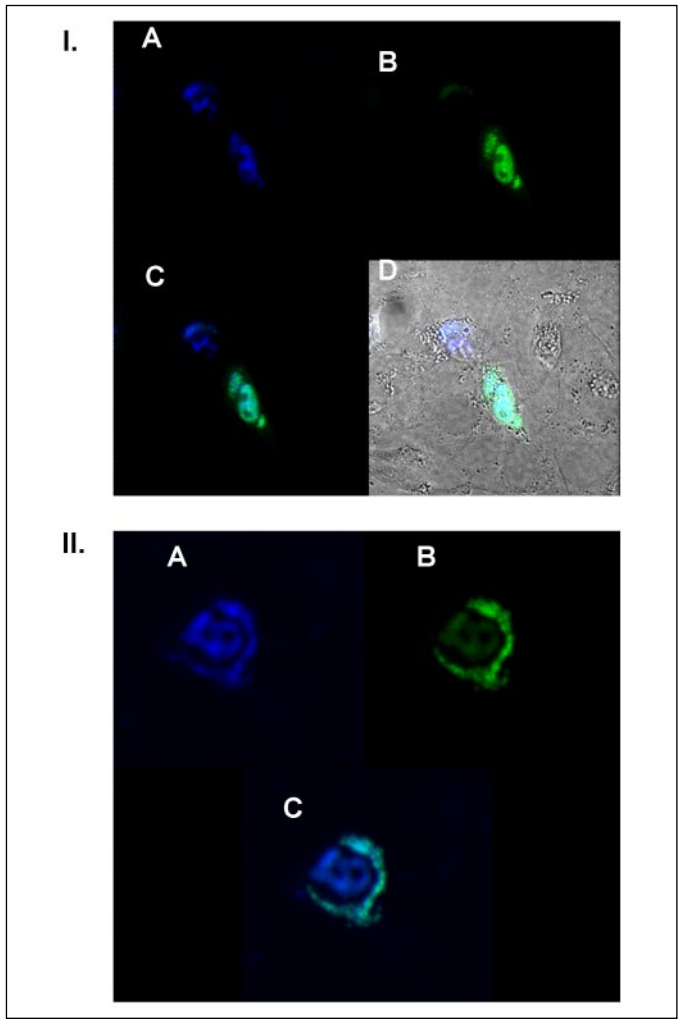

FIGURE 7. **Co-transfection and localization of SECp43 and HSLA.** Confocal images of NIH3T3 cells co-transfected with SEC43-BFP and HSLA-GFP photographed 48 h post-transfection are shown as follows. *I*, SECp43-BFP (*A*), HSLA-GFP (*B*), merged blue and green signals (*C*), and merged signals including phase contrast (*D*) (40× magnification); *II*, SECp43-BFP (*A*), HSLA-GFP (*B*), merged signals of BFP and GFP (*C*) (60× magnification).

The same result was obtained using a GFP·HSLA fusion construct (data not shown).

Because the co-immunoprecipitation studies with SECp43 and MSLA revealed that they formed a complex, we examined the co-transfection of the SECp43-BFP fusion and the HSLA-GFP fusion proteins to assess their subcellular co-localization. As shown in Fig. 7, after co-transfecting NIH3T3 cells with the corresponding fusion protein constructs, HSLA appeared in the nucleus. As SLA does not have an obvious nuclear localization signal or phosphorylation sites that might govern its transfer to the nucleus, this finding suggested that SECp43 may act as a chaperone for SLA directing its subcellular localization. These observations are further considered below in the Discussion.

DISCUSSION

When both SECp43 (16) and SLA (17) were discovered, they were characterized as RNA-binding proteins that formed a complex with Sec tRNA$^{[Ser]Sec}$. SECp43 was also found to have two RNA binding sites and to interact with a 48-kDa protein. The 48-kDa component, however, was not identified (16). In a separate study, a 48-kDa protein was precipitated in a complex with an autoantibody in human cell extracts that was found in patients with autoimmune chronic hepatitis (17). It is apparent from the present study that the SECp43-associated protein was most likely SLA as MSLA is a 48-kDa protein, and it was found herein to exist in a complex with SECp43 in NIH3T3 cells. Interestingly, Sec tRNA$^{[Ser]Sec}$ was also shown to occur in this complex providing strong evidence that the multifarious complex includes all three components. More importantly, our study provided the first evidence for the role of SECp43 and SLA in regulation of selenoprotein expression and firmly linked these proteins to the pathway of selenoprotein biosynthesis.

Different approaches were employed to elucidate the function of the two protein components in the complex. Initially, we used RNAi technology to knockdown SECp43 mRNA and devised a siRNA vector encoding five separate targeting sites in MSLA mRNA to effectively reduce its level. Under our cell culture conditions, knockdown of SECp43 mRNA had no effect on MSLA mRNA expression, and the knockdown of MSLA mRNA had no effect on SECp43 expression in either NIH3T3 or TCMK-1 cells. Although the virtual removal of MSLA mRNA had no detectable effect on selenoprotein expression, knockdown of SECp43 mRNA affected GPx1 mRNA and protein expression levels. Because these observations regarding GPx1 mimic those observed in mammalian cells and tissues containing reduced levels of the mcm^{5}Um isoform (4, 5), we examined the effect of SECp43 knockdown on the distribution of mcm^{5}U and mcm^{5}Um in NIH3T3 cells. The level of mcm^{5}Um relative to mcm^{5}U was reduced in cells transfected with the SECp43 siRNA construct compared with cells transfected with the control vector. These studies provide evidence that SECp43 is involved in Um34 methylation. Whether this is a transient effect on Um34 synthesis or a direct effect that is responsible for Um34 formation must await further study. However, additional studies on SECp43 described herein clearly show that it has other roles than an involvement in methylation of Sec tRNA$^{[Ser]Sec}$ at position 34 (see below).

Interestingly, simultaneous knockdown of SECp43 and MSLA mRNAs reduced the overall expression of selenoproteins suggesting that the two protein factors act in concert. The two factors do indeed

form a complex with Sec tRNA$^{[Ser]Sec}$, and the targeted reduction of either one of the factors substantially reduced the overall binding of tRNA to the complex. The cellular location of SECp43 suggests additional roles of this factor. For example, SECp43 occurs primarily in the nucleus; however, when the construct encoding it was co-transfected with that of SLA, it resulted in the nuclear translocation of SLA from the cytoplasm to the nucleus. This observation suggests that SECp43 may function as a chaperone for SLA and Sec-tRNA$^{[Ser]Sec}$. Although the present study provides new insights into the roles of SECp43 and SLA and firmly plants them as important factors in the biosynthesis of Sec and its incorporation into protein, the full characterization of these two factors must await further studies.

Sec insertion in bacteria has been thoroughly characterized and all involved factors identified (28). The bacterial pathway includes an elongation factor (SelB), Sec synthase (SelA), Sec tRNA (SelC), and selenophosphate synthetase (SelD). Conservation of some of these components suggested that a similar mechanism operates in eukaryotes, including mammals. However, recent studies clearly changed this paradigm as several additional factors have been identified (1, 2). The present study has demonstrated the significance of two additional factors, SECp43 and SLA, in this pathway that are not present in bacteria. These observations highlight an increasing complexity of the Sec insertion machinery during evolution.

In conclusion, this study provides the first evidence for the direct role of SECp43 and SLA in selenoprotein biosynthesis. These factors interact and occur in a complex with tRNA$^{[Ser]Sec}$. In addition, our study linked SECp43 to the regulation of selenoprotein synthesis through its involvement in methylation of tRNA$^{[Ser]Sec}$ and the intracellular distribution of SLA.

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