Selenoprotein P Expression, Purification, and Immunological Characterization*

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Most selenoproteins contain a single selenocysteine residue per polypeptide chain, encoded by an in-frame UGA codon. Selenoprotein P is unique in that its mRNA encodes 10–12 selenocysteine residues, depending on species. In addition to the high number of selenocysteines, the protein is cysteine- and histidine-rich. The function of selenoprotein P has remained elusive, in part due to the inability to express the recombinant protein. This has been attributed to presumed inefficient translation through the selenocysteine/stop codons. Herein, we report for the first time the expression of recombinant rat selenoprotein P in a transiently transfected human epithelial kidney cell line, as well as the endogenously expressed protein from HepG2 and Chinese hamster ovary cells. The majority of the expressed protein migrates with the predicted 57-kDa size of full-length glycosylated selenoprotein P. Based on the histidine-rich nature of selenoprotein P, we have purified the recombinant and endogenously expressed proteins using nickel-agarose affinity chromatography. We show that the recombinant rat and endogenous human proteins react in Western blotting and immunoprecipitation assays with commercial anti-histidine antibodies. The ability to express, purify, and immunologically detect the recombinant protein provides a foundation for investigating the functions and efficiency of expression of this intriguing protein.

Selenocysteine, the 21st amino acid, is cotranslationally incorporated into selenoproteins at UGA codons, which typically function as stop codons. This rare amino acid is found in the active site of all selenoenzymes characterized to date. Most selenoprotein mRNAs contain a single UGA codon encoding a single selenocysteine residue per polypeptide chain and a single specific RNA secondary structure, termed a SECIS element, directing incorporation of this amino acid. Selenoprotein P is unique in that its mRNAs in different mammalian species encode 10–12 selenocysteine residues and contain two SECIS elements in the 3′-untranslated region (1–4).

Selenoprotein P is a glycoprotein that carries up to 50% of the selenium in plasma, but its function is unknown. Although originally identified in plasma and shown to be expressed in liver, more recent studies have identified selenoprotein P mRNA in a wide variety of tissues. An abundance of selenoprotein P message is present in specific regions of the brain, in kidney, testis, lung, and heart (4–6). Selenoprotein P cDNA sequences in the GenBank™ data bases indicate expression in human thyroid, prostate, a T cell lymphoma, and an endometrial tumor, as well as in mouse myotubes and mammary gland. Response elements for hepatic nuclear factor, HNF3, and a brain-specific transcription factor, BRN-2, are present in the murine gene promoter (7), consistent with expression of the mRNA in these tissues. Expression of selenoprotein P has been shown to be repressed by cytokines in HepG2 cells (8) and induced by dexamethasone treatment in normal rat kidney cells (9), suggesting possible roles in inflammatory responses and repression during the acute phase reaction.

Immunohistochemical localization of selenoprotein P revealed its presence in the liver and brain bound to capillary endothelial cell walls. In kidney, the protein is found associated with the linings of the glomerulus but not in the tubules (10). In vivo, selenoprotein P is thought to serve as an antioxidant in protection from diquat-induced liver necrosis (11, 12). Observed in vitro functions include exhibiting a low level extracellular phospholipid hydroperoxide glutathione peroxidase-like activity (13), promoting survival of neurons in primary culture (14), and binding to complexes consisting of equimolar ratios of heavy metals and selenium, i.e. Hg-Se, Cd-Se, and Ag-Se (14–16). The protein has also been proposed to function in selenium transport or storage (6). The localization of selenoprotein P to the capillary endothelial cells may correlate with protection from products of oxidative stress or as a barrier to heavy metal uptake by cells.

In addition to the presence of 10–12 selenocysteines, the protein has a high content of cysteine and histidine residues. The rat and human selenoprotein P sequences each encode two histidine-rich regions; the first region consists of 8 (rat) or 9 (human) histidines out of 14 residues, and the second a stretch of 7 (rat) or 4 (human) consecutive histidines. These regions, in conjunction with the cysteine and selenocysteine content, are likely responsible for the observed coordination to heavy metals (17). This property has been utilized in designing affinity purification strategies, employing either zinc-Sepharose (14) or nickel-agarose (16).

All studies to date reporting characterization or functional studies of selenoprotein P have relied on protein expressed endogenously by various cell lines or purified from plasma or serum. Progress has been made in defining the biochemical properties, such as heavy metal binding, and correlation with physiological effects, e.g. antioxidant activity and extension of neuronal survival. However, the function(s) and particularly the mode of action of selenoprotein P remain elusive, in part due to the lack of a transgenic or knockout model and the reported inability to express the recombinant protein in mammalian cells. Similarly, little is known about the efficiency of selenocysteine incorporation into selenoprotein P, either in cell...
lines or in the intact animal. Studies in transfected mammalian cells (18) and in bacteria (19) suggest that selenocysteine incorporation at a single site per polypeptide may be inefficient. If so, this raises the question of how the translation machinery produces full-length selenoprotein P, outcompeting termination at 10–12 UGA codons.

Herein, we describe expression of recombinant rat selenoprotein P in a transiently transfected human epithelial kidney cell line. We show that the expressed rat protein is glycosylated to a similar extent as human and hamster selenoprotein P expressed endogenously in cell lines from these two species. Taking advantage of the histidine-rich nature of the proteins, we utilized nickel-agarose affinity chromatography to purify the expressed recombinant protein. We show that commercially available antibodies prepared against 4 or 5 consecutive histidines detect the expressed protein in both Western blotting analysis and immunoprecipitation assays. The reactivity of these antibodies with unlabeled selenoprotein P in Western blots thus circumvents the need to use radiolabeled selenium to study the protein. These results provide an excellent foundation for investigation of the functions of selenoprotein P, as well as the mechanism and efficiency of selenocysteine incorporation into this protein.

**EXPERIMENTAL PROCEDURES**

** Constructs—**The rat selenoprotein P cDNA was a generous gift of Kristina Hill (Vanderbilt University, Nashville, TN). Two upstream PCR primers (Life Technologies Inc.), GGAATTCCGCAATGTGTGAGAACCTAGGGCTTG and GGAATTCCACACATGGGGAGAAGAGCCTAGGTG, were designed corresponding to the region of the initiation codon (italics). One amplifies the wild-type sequence, in which the ATG codon (underlined) just upstream of the coding region. A downstream PCR primer complementary to nucleotides 2550–2532 in the 3'-untranslated region and encoding a RI site and a XhoI site was used in conjunction with the upstream primers to amplify the selenoprotein P coding and 3'-untranslated region sequences. The ~2.3-kilobase pair PCR products were subcloned into pUHD10-3 vector (20) via the unique XbaI site. A second primer was designed to create a favorable Kozak context (ACC ATG T). These primers introduce an EcoRI site (underlined) just upstream of the coding region. A downstream PCR primer complementary to nucleotides 2550–2532 in the 3'-untranslated region and encoding a NotI site was used in conjunction with the upstream primers to amplify the selenoprotein P coding and 3'-untranslated region sequences. The ~2.3-kilobase pair PCR products were subcloned into pHUD10-3 vector (20) via the unique EcoRI site and a NotI site introduced adjacent to the XhoI site. Constructs were verified by dideoxy sequencing to ensure that no mutations were introduced by PCR.

**CELL CULTURE CONDITIONS AND TRANSIENT TRANSFECTIONS, HEK293 CELLS**—Transient transfections in human embryonic kidney (HEK) 293 cells were carried out using the calcium phosphate method of transfection as described previously (21). Three days prior to transfection, cells were plated onto 60-mm culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with 10 μg of the pUHD10-3 based expression plasmids and 4 μg of the pUHD15 plasmid (20), which encodes a protein necessary for transcriptional activation of the pUHD10-3 promoter. To monitor transfection efficiencies, cells were cotransfected with 3 μg of an expression vector containing the human growth hormone cDNA under control of the herpes simplex virus-thymidine kinase promoter. On the day of transfection, media were changed to DMEM supplemented with 1% FBS. The day following transfection, media were changed to DMEM without serum to reduce the amount of albumin in the preparations. For labeling studies, 35S-20% (w/v) bovine serum albumin was added to the media at this time. Two days after transfection, media and cells were harvested. Cells were washed in phosphate-buffered saline and resuspended in 0.1 M potassium phosphate, pH 6.9, 1 mM EDTA containing 0.25 M sucrose. For studies of the effects of selenium supplementation, graded amounts of sodium selenite were added to media the day before transfection.

**HepG2 and CHO Cells—**HepG2 human hepatoma cells were maintained in DMEM supplemented with 10% FBS. Chinese hamster ovary (CHO) cells were maintained in a 1:1 mixture of DMEM and Ham’s F-12, supplemented with 10% FBS. Transfection with DEAE-dextran was performed according to the procedure described (22). DEAE-dextran was prepared in phosphate-buffered saline supplemented with 0.1 mM chloroquine. Media were changed to DMEM without serum at the time of addition of 75Se as above, and labeling was continued for 24 h.

**PEPTIDE FRACTIONATION AND Western Analysis**—Proteome preparation from transiently transfected HEK 293 cells was carried out according to a modification of the protocol described by White et al. (23). Cells were harvested as above, except that cycloheximide was added to 100 μg/ml 20 min before harvest. Cells combined from three 100-mm dishes were washed in PBS buffer and resuspended in low salt buffer (LSB, 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM MgCl2) containing 1 mM RNasin, 1 mM dithiothreitol. Cells were homogenized in a Dounce homogenizer in LSB containing 1.2% Triton X-100 and 0.25 M sucrose. Postmitochondrial supernatants were prepared by centrifugation in a benchtop microcentrifuge at 12,000 × g for 5 min. NaCl and MgCl2 concentration were adjusted to 150 and 10 mM, respectively. Linear sucrose gradients (15–50% w/w in LSB) were prepared using a Searle Densi-flow ICE gradient maker with a sublayer of 1 ml of Fluorinert (ISCO, Lincoln, NE). Cytoplasmic extracts (1 ml) were layered onto 10.6-m1 gradients and centrifuged at 150,000 × g for 2 h. Fractions (0.75 ml) were collected using a gradient fractionator (Brandel) equipped with an ISCO UA-6 UV detector. Polysome profiles were detected by absorbance at 294 nm. RNA was isolated from 250 μl of each gradient fraction using the TRI REAGENT LS (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s specifications. RNAs were electrophoresed on denaturing agarose gels and vacuum-blotted to Nitran membranes (Schleicher & Schuell). A DNA fragment from the selenoprotein P cDNA (Ngel fragment, 783 base pairs) was labeled by random priming (Random Primer Labeling Kit, Stratagene). Hybridization was carried out at 65 °C followed by high stringency washing at 65 °C. Protease and Proteasome Inhibitors—Protease or proteasome inhibitors were added to media at the time of 75Se labeling. CompleteT protease inhibitor mixture (Roche Molecular Biochemicals) containing inhibitors of chymotrypsin, thermolysin, papain, Pronase, and trypsin was used according to the manufacturer’s specifications. N-Acetyl-l-leucyl-l-leucyl-l-norleucinal (AALLN, Sigma), a calpain inhibitor (24), was added to media at a final concentration of 50 μM. Carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (CMLL), a proteasome uptake inhibitor (25), was added at a final concentration of 10 μM.

**Deglycosylation—**Deglycosylation was performed using the N-Glycosidase F Deglycosylation Kit from Roche Molecular Biochemical according to the manufacturer’s specifications. Nickel-Agarose Affinity Chromatography—Nickel-agarose chromatography was performed using nickel-nitrilotriacetic acid and agarose beads (Qiagen, Valencia, CA) or Bio-Rad poly-prep columns (2-ml bed volume) filled with nickel-nitrilotriacetic acid columns. Columns were equilibrated with protein binding (PB) buffer, consisting of 20 mM Tris-HCl, pH 8.0, 0.3 M NaCl; 10 mM β-mercaptoethanol, 10% glycerol. Media from transfected cells (up to 3 columns) were allowed to percolate into the columns by gravity. Column resin was washed at least 3 times with PB buffer containing typically 0.5 M NaCl and proteins were eluted by washing columns with PB buffer containing 0.5 M NaCl and 100–400 mM imidazole. If necessary, proteins were concentrated in Viva-spin columns (VivaScience, Lincoln, UK).

**Immunoprecipitation—**Tetra-histidine- and penta-histidine-specific antibodies were obtained from Qiagen. Primary antibodies were diluted according to the manufacturer’s specifications. Typically 5 μl were incubated with 300 μl of selenium-labeled media from cells. Immunoprecipitation was carried out according to Ref. 41 except that Pansorbin (Calbiochem) was used as a source of protein A. Samples were boiled for 5 min in SDS sample buffer containing 0.5 μl β-mercaptoethanol, and electrophoresed on 10% acrylamide gels (acrylamide:bisacrylamide, 29:1) or 12% ReadyGels (Bio-Rad). Samples were subjected to SDS-PAGE analysis on 5 or 12% polyacrylamide gels (acrylamide:bisacrylamide, 29:1) or 12% ReadyGels (Bio-Rad). Samples were subjected to SDS-PAGE analysis on 10 or 12% polyacrylamide gels (acrylamide:bisacrylamide, 29:1), followed by electrotransfer to Immobilon membranes (Millipore, Bedford, MA) in 20% methanol, 25 mM Tris-HCl, pH 8.3, 192 mM glycine. Membranes were blocked with 3% (w/v) bovine serum albumin in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), incubated with histidine-specific antibodies (Qiagen) at 1:1500 dilution in 3% (w/v) bovine serum albumin (BSA) and then washed in TBS/Tris/Tween according to the manufacturer’s instructions. This was followed by incubation with goat anti-mouse horseradish peroxidase-conjugated secondary antibody in 10% (w/v) nonfat milk in TBS (16000 dilution, Roche Molecular Biochemicals). Reaction products were visualized by enhanced chemiluminescence (Roche Molecular Biochemicals) and exposure to X-OMat film (Eastman Kodak Co., Rochester, NY). After extensive washing of the
Expression of Recombinant and Endogenous Selenoprotein P in Mammalian Cell Lines—We hypothesized that one possible reason for the reported inability to express selenoprotein P in transfected cells was due to the unfavorable context, GCA ATG T, of the initiation codon in the wild-type (w.t.) rat selenoprotein P sequence. To test this hypothesis, the w.t. sequence was mutated to the Kozak consensus sequence (26), ACC ATG G. W.t. and mutant selenoprotein P expression constructs were then transiently transfected into HEK 293 cells using the calcium phosphate precipitation method. We chose to visualize the expression of selenoprotein P first by polysome analysis. Determination of the number of ribosomes associated with an RNA reflects its translational activity. Cytoplasmic extracts were prepared and loaded onto sucrose gradients for polysome isolation and fractionation. The A260 profile of a representative gradient is shown in Fig. 1. RNA was prepared from each gradient fraction and analyzed by Northern hybridization to localize selenoprotein P mRNA in the gradient. Fig. 1 gradient fraction and analyzed by Northern hybridization to localize selenoprotein P mRNA in the gradient. RNA was prepared from each fraction of the profile shown above, using a selenoprotein P probe. kb, kilobase pair.

RESULTS

Expression of Recombinant and Endogenous Selenoprotein P

Since selenoprotein P mRNA appeared to be translated efficiently, we analyzed media from cells transiently transfected with the selenoprotein P expression plasmid and labeled with 75Se. Forty-eight hours after transfection and after a 24-h labeling period, 75Se-labeled selenoprotein P was present in the media from transfected cells (Fig. 2, lane 2). The recombinant protein migrated with the observed molecular mass of full-length, or near full-length selenoprotein P, ~57 kDa. No endogenously expressed selenoprotein P was detectable in the media from HEK cells following transfection with vector alone (Fig. 2, lane 1). We also analyzed the labeled selenoprotein profile in cell lysates. We were unable to detect any differences in the cell lysate labeling pattern between the selenoprotein P and vector-transfected cells. Thus, no new selenoprotein bands corresponding to the sizes of prematurely terminated or non-glycosylated selenoprotein P species were seen in cell lysates following transfection. However, the high background of endogenous selenoproteins in the size range of the full-length protein, including thioredoxin reductases and selenophosphate synthetase, might easily mask full-length or near full-length glycosylated selenoprotein P associated with cells (see below). Endogenous selenoproteins in the size range corresponding to cytoplasmic and phospholipid hydroperoxide glutathione peroxidase were also present in these cells.

We next investigated the abilities of other cell lines to express the recombinant rat protein. Following 75Se labeling of CHO cells, endogenously expressed secreted selenoprotein P was easily detected in the media (Fig. 2, lane 3). However, the expression of recombinant selenoprotein P was negligible, as there was no increase in the amount of selenoprotein P observed after transfection of the plasmid encoding the rat protein (not shown). The human hepatoma cell line, HepG2, is known to express several selenoproteins, including type I deiodinase2 and selenoprotein P (5). Following 75Se labeling, HepG2 cells also produced endogenous selenoprotein P (Fig. 2, lane 4) but no additional selenoprotein P upon transfection of the plasmid (not shown). Strikingly, the patterns of labeled proteins differed from these three cell lines. Transfection of the rat cDNA into HEK 293 cells produced a single prominent selenoprotein migrating as ~57 kDa, whereas the human protein produced endogenously by HepG2 cells migrated slightly more slowly, in the 60-kDa size range. As the deduced molecular masses only differ by ~170 Da, this may reflect differences in extent of glycosylation. In CHO cells, a faster migrating selenoprotein of ~45 kDa was also observed, in addition to the ~57-kDa protein. This may correspond to a prematurely terminated form of the protein (see “Discussion”).

One potential difficulty in determining the extent of premature termination of endogenous or recombinant expressed selenoprotein P from these cell lines is that these products may be rapidly degraded within the cell, underestimating the level of termination. This has been shown to be the case for the UGA-terminated product produced upon transfection of type 1 deiodinase cDNA in HEK cells (see below). To address this question, we examined the effects of adding protease or proteasome inhibitors to cells during in vivo 75Se labeling. We tested the calpain I-specific protease inhibitor, ALLN (Sigma), which stabilizes the type 1 deiodinase UGA-termination product.3 Addi-

2 R. M. Tujebajeva, J. W. Harney, and M. J. Berry, unpublished observations.
3 G. Warner, personal communication.
tion of either ALLN or a protease inhibitor mixture, Complete™ (Roche Molecular Biochemicals), had no detectable effect on the patterns of selenoproteins either in the media or in cell lysates (not shown) from any of the three cell lines. Similarly, the proteasome uptake inhibitor, MG132, did not affect the selenoprotein patterns.

As selenium may be limiting for expression of selenoprotein P, we examined the effects of supplementation of media with unlabeled selenium. Sodium selenite was added to a final concentration of 0, 10, 30, or 100 nM the day before transfection. The selenium concentration in the “0 added” condition is estimated at less than 1 nM. Thus, the increase in unlabeled selenium to 100 nM results in a >100-fold decrease in specific activity. Supplementation with 10, 30, or 100 mM selenium led to 1.2-, 3.6-, and 7.2-fold increases, respectively, in labeling intensity versus the unsupplemented lane (data not shown). Examination of the labeling intensity over this range revealed a progressive increase in incorporation of label into selenoprotein P despite the large decrease in specific activity, indicating a dramatic increase in selenoprotein P production. No apparent changes in the ratios of individual labeled bands were detected with the increase in selenium concentration. In addition, no detectable differences in cell growth or morphology were observed during the time course of the experiment.

**Glycosylation Patterns of Recombinant and Endogenously Expressed Selenoprotein P**—Previous studies have shown that rat selenoprotein P undergoes N-linked glycosylation in at least three of the five potential sites (27). To determine if some of the observed differences in migration patterns could be accounted for by differences in glycosylation, selenoprotein P preparations were treated with endoglycosidase F. A decrease in the apparent molecular weights of the selenium-labeled proteins from all three cell lines was observed, indicating that the protein is glycosylated in all three lines (Fig. 3). The mobility of the HepG2 human protein decreased from an apparent molecular mass of ~60 to ~45 kDa and the recombinant rat protein from ~57 to ~45 kDa. The larger and smaller selenoprotein species from CHO cells (~57 and ~45 kDa) decreased to ~38 and ~26 kDa following endoglycosidase treatment, representing changes of ~19 kDa. This latter change indicates that the two forms produced by CHO cells are probably glycosylated to a similar extent (Fig. 3, lane 3 versus 4).

Although the majority of selenoprotein P was expected to be in the media, and no premature termination products or deglycosylated forms were detected in the cell lysate, some glycosylated selenoprotein P might be present in the cell lysate fraction, due to either association with the outer cell membrane or perhaps in intracellular compartments in the process of being secreted. Glycosylated selenoprotein P would be predicted to comigrate with other endogenous selenoproteins, making it difficult to detect. We might be able to reveal its presence by deglycosylation, thus increasing its mobility relative to the other endogenous selenoproteins. Lysates from HEK cells transfected with the selenoprotein P plasmid or empty vector were treated with endoglycosidase F and analyzed by SDS-PAGE in parallel with untreated lysates and treated and untreated media. Upon overexposure of the autoradiogram, a very faint band, corresponding in size to the major selenoprotein P band in endoglycosidase-treated media, was detected in the endoglycosidase-treated selenoprotein P-transfected lysate (data not shown). This band was not seen in the endoglycosidase-treated vector-transfected lysate. Thus, the only selenoprotein P-specific band detectable in cell lysates corresponds to full-length or near full-length protein.

**Purification of Selenoprotein P by Nickel-Agarose Affinity Chromatography**—As previous studies have utilized nickel-agarose or zinc-Sepharose chromatography to purify selenoprotein P from serum, we wanted to determine if the recombinant selenoprotein exhibited similar chromatographic behavior. Media were harvested from HEK 293 cells following transfection of the rat selenoprotein P plasmid and 75Se labeling. Media (Fig. 4, lane 1) were percolated through a bed of nickel-agarose resin, the flow-through fraction collected (lane 2), and the resin washed with buffer containing 0.5 M NaCl to elute nonspecifically bound proteins (Fig. 4, lanes 3–5). This was followed by washes in the same buffer containing 100, 200, or 400 mM imidazole to elute specifically bound protein (lanes 6–8). The majority of the labeled protein eluted in the 100 mM imidazole fraction (Fig. 4, lane 6). The resin was subsequently washed with buffer containing 400 mM imidazole and 20 mM EDTA to strip nickel from the column. No additional labeled protein was eluted with this treatment (Fig. 4, lane 9). We next tested the column resin for the presence of tightly bound protein. Column resin was boiled in electrophoresis sample buffer containing SDS and β-mercaptoethanol, and an equivalent fraction was analyzed by SDS-PAGE. This revealed that a small fraction of the selenoprotein P was tightly bound to the nickel-agarose resin (Fig. 4, lane 10). Chromatography of media from 75Se-labeled HepG2 or CHO cells resulted in elution profiles similar to that observed with HEK 293 cell media (not shown).

**Immunoprecipitation with Commercial Polyhistidine-specific Antibodies**—Based on the presence of consecutive histidine residues in the rat and human selenoprotein P sequences, these proteins would be predicted to react with antibodies prepared against tetra-histidine or penta-histidine peptides. The antibodies were analyzed for their ability to immunopre-
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FIG. 5. Immunoprecipitation of selenoprotein P with anti-histidine antibodies. Lanes 1 and 2, transfected HEK 293 cell media; lanes 3 and 4, CHO cell media; lanes 5 and 6, HepG2 cell media. Odd-numbered lanes, penta-histidine antisera; even-numbered lanes, tetra-histidine antisera.

FIG. 6. Western analysis of selenoprotein P with anti-histidine antibodies. A, Western blot of proteins purified from media by nickel-agarose chromatography. Penta-histidine antibodies were used in the figure. Lane 1, protein purified from transfected HEK 293 cell media; lane 2, protein purified from CHO cell media; lane 3, protein purified from HepG2 cell media. B, autoradiograph of the blot shown in A, following decay of the chemiluminescent signal.

Describes the visualization of the 75Se-labeling patterns of the purified protein with antibodies. The slow moving band, which predominated in media (Fig. 6B, lane 1 versus Fig. 3, lane 1), in following the decay of the chemiluminescent signal, the Western blot membrane was exposed for autoradiography to allow visualization of the 75Se-labeling patterns of the purified proteins. Comparison of this autoradiogram with those in Figs. 2 and 3 revealed differences in patterns of 75Se-labeled bands in the nickel-agarose-purified material versus the patterns present in media. Following purification of the protein from the HEK cell media, the faster migrating band was enriched relative to the slower migrating band, which predominated in media (Fig. 6B, lane 1 versus Fig. 3, lane 1). In material purified from HepG2 cell media, two prominent, closely spaced 75Se-labeled bands appear, whereas only one was apparent in the media (Fig. 6B, lane 3 versus Fig. 2, lane 4, and Fig. 3, lane 5). These differences may result from enrichment of specific isoforms of selenoprotein P by the chromatography step. The autoradiogram also confirmed that the amount of purified CHO selenoprotein in the membrane was comparable to the amounts of the proteins from the other two sources, indicating that insufficient material could not explain the lack of detection by antibody (Fig. 6B, lane 2). Finally, Fig. 6B revealed that while some of the selenium-labeled bands in the HepG2 and HEK lanes correspond with antibody-reactive bands in Fig. 6A others do not. Possible explanations for these results will be discussed below.

DISCUSSION

Until now, attempts to express recombinant selenoprotein P in mammalian cells have been unsuccessful. We have previously shown that expression of high levels of another selenoprotein, type 1 iodothyronine deiodinase, results in inefficient readthrough and high levels of termination in mammalian cells (18). Similar results have been reported for attempts at selenoprotein expression in the baculovirus system (28). This may be due to titration of the endogenous components of the selenoprotein synthesis machinery, including selenocysteinyl-tRNA and the putative selenocysteine-specific elongation factor, by excess selenoprotein mRNA. These effects would be particularly pronounced for selenoprotein expression from efficiently transfected and transcribed plasmids. Alternatively, an inherent inefficiency in the translation process, e.g. selenocysteine incorporation being a slow elongation step, may contribute to high levels of termination. This has recently been shown to be the case for selenoprotein synthesis in Escherichia coli (19). If selenocysteine incorporation were inefficient in a protein with a single selenocysteine residue per polypeptide, we might predict an even lower efficiency for a protein containing multiple selenocysteine residues. Surprisingly, in this study we obtained efficient production of selenoprotein P in transfected HEK cells. The level of expressed recombinant protein appears to be at least comparable to endogenous selenoprotein P production by HepG2 cells. The ability to express the protein in HEK cells is likely due in part to the high efficiency of transfection capable in this cell line, between 10- and 100-fold higher than most other lines we've studied. The lack of increased expression following transfection of HepG2 and CHO cells may be due to lower transfection efficiencies in these cell lines. The majority of either recombinant or endogenously expressed selenoprotein P migrates with the predicted mobility of full-length or near full-length protein, indicating efficient incorporation at multiple UGA codons within an mRNA in all three cell lines. Small amounts of truncated protein present in media from transfected but not vector control cells likely represent selenoprotein P premature termination products (see below).

Increasing the amount of unlabeled selenium in the media
dramatically increased the amount of selenoprotein P produced but had no apparent qualitative effect on the labeling pattern, in contrast to results previously reported by Himeno et al. (27) from in vivo studies (see below). This may be due to a combination of several factors. Selenium may have been quite limiting at the lower concentrations, resulting in significant termination at the first UGA codon, the product of which would not be detectable by labeling or with the histidine-specific antibodies. Termination at some of the other UGA codons might result in unstable isoforms that would be rapidly degraded and thus not detected. An increase in selenium concentration, increasing the total pool of charged selenocystyl-tRNA, would lead to more full-length selenoprotein P, which might be more resistant to degradation. In addition, selenium supplementation has been shown to shift the distribution of the tRNAsec population, increasing the levels of a specific tRNA methylation, and to decrease the turnover rate of tRNAsec, further increasing selenocysteine incorporation efficiency (29). Stabilization of the tRNA may be a direct result of the modification. There may also be preferential incorporation of selenium into selenoprotein P versus other selenoproteins at the higher selenium levels. Finally, there may be effects on cellular metabolic rate, but these are likely to be minor during the short time of incubation with graded selenium concentrations, and no such effects were apparent in these studies.

As the only secreted selenoproteins reported to date are selenoprotein P and plasma glutathione peroxidase (molecular mass ~21 kDa), we are intrigued by the presence of multiple 76Se-labeled bands in the media and the nickel-agarose purified preparations from the three cell lines studied, as well as variations between the crude and purified material. The fact that these bands all contain selenium and are secreted into the media and that in the purified material they copurify on nickel-agarose supports their assignment as isoforms of selenoprotein P, most likely consisting of alternatively modified and/or folded forms or UGA termination products. Himeno et al. (27) previously reported identification of different isoforms of selenoprotein P in rat plasma. These isoforms consist of protein undergoing termination at the second UGA codon, confirmed by carboxyl-terminal sequencing, as well as presumed full-length protein. Termination at the first selenocysteine codon would not have been detected in these studies, as 76Se labeling was used to identify the proteins. The amount of protein terminated at the second UGA was increased in selenium-deficient animals, indicating that limiting selenium influenced the choice between incorporation and termination. Similarly, immunoaffinity purification of selenoprotein P from human plasma revealed two bands on SDS-PAGE with mobilities corresponding to ~61 and 55 kDa (30). Comparison of the available selenoprotein P sequences reveals the proteins from different species to be highly homologous. The positions of most of the selenocysteine codons are conserved, but there are some substitutions of cysteine for selenocysteine or vice versa between species. The second UGA codon is not conserved between species. In the rat sequence, it is codon 245, whereas in the human sequence, the second UGA doesn’t appear until codon 281. Thus, the truncated selenoproteins resulting from premature termination at these codons would be predicted to differ in size. The hamster selenoprotein P sequence is unavailable; thus we cannot predict whether the smaller selenoprotein produced in CHO cells would likely result from termination at an early UGA codon and, if so, which one. However, the size of the smaller deglycosylated selenoprotein from CHO cells (~26 kDa) corresponds closely with that predicted for termination at the second UGA codon in the rat sequence (~27.5 kDa). In both the rat and human sequences, the 9th and 10th selenocysteine codons are clustered within the last 6 codons very near the carboxyl terminus, and the 7th and 8th selenocysteine codons reside within the last 15 codons of the open reading frame. Amino acid composition of the putative full-length protein purified by Himeno et al. (27) revealed the presence of only 7.5 mol of selenium per mol of peptide, instead of the predicted 10, suggesting possible heterogeneity. Furthermore, isolation of tryptic peptides failed to reveal the carboxyl-terminal peptide predicted to contain the last 4 selenocysteines. Thus, the putative full-length protein purified by these investigators, as well as the protein expressed herein, could consist of a mixture of full-length selenoprotein P and near full-length selenoprotein P resulting from termination at any of these last 4 UGA codons. These differences could affect the way the proteins migrate on SDS-PAGE, how they fold or refold following renaturation, and whether the histidines are accessible to antibodies. This could in turn explain the differences in patterns detected by selenium labeling and autoradiography versus Western blotting in Fig. 6.

There are six potential glycosylation sites in the human selenoprotein P sequence and five in the rat sequence. Of these, two are conserved between these two species, as well as in the murine and bovine sequences. Three of the five potential glycosylation sites in the rat sequence have been shown to be used (27). The predicted sizes of human and rat selenoprotein P without carbohydrate are 41,229 and 41,052 Da, respectively. Thus, the ~3-kDa difference in apparent sizes of the rat and human proteins on SDS-PAGE likely derives either from differences in glycosylation patterns or other modifications or in protein folding or Stokes radius. The sizes of the recombinant and endogenously expressed proteins show close agreement with the sizes previously reported for selenoprotein P purified from rat, human, or bovine plasma (13, 14, 27). After deglycosylation, the mobilities also correlated with those previously reported, and with the molecular weights predicted from the cDNA sequences.

Based on sequence, we would predict that rat selenoprotein P would react with both the tetra- and penta-histidine antibodies, due to the presence of seven consecutive histidines. However, the human sequence has four consecutive histidines but, unexpectedly, reacts with the penta- as well as the tetra-histidine antibody. Thus it is somewhat surprising that the rat protein reacted only weakly with the tetra-histidine antibody. Without the hamster sequence, we cannot predict whether the lack of detection of CHO protein by antibodies in Western blotting or immunoprecipitation reactions is due to the lack of a sufficient number of consecutive histidine residues. Nonetheless, the ability to detect both the rat and human protein without the use of radioisotopes represents a valuable tool for studying the function of these proteins.

One of the most extensively studied roles for selenoprotein P is in protection from diquat-induced liver necrosis. Following selenium depletion and subsequent repletion, the reappearance of selenoprotein P, but not of other selenoproteins such as glutathione peroxidase, correlates with resistance to diquat toxicity (11). A recent report indicated that selenoprotein P possessed phospholipid hydroperoxide glutathione peroxidase activity (13), suggesting a direct antioxidant role for this protein. However, the reported activity was 2 orders of magnitude lower than that of the cellular enzyme, and the possibility of contamination by other enzymes was not ruled out. Co-administration of selenium has long been known to play a role in reducing the toxic effects of mercury (31, 32). Based on the demonstrated ability of selenoprotein P to bind to heavy metals (15, 33) and to Hg-Se complexes in serum (34), a role for selenoprotein P in detoxifying these heavy metals has been proposed. A metal-responsive element, MRE2, is present in the
bovine gene, but no induction was observed following treatment with micromolar concentrations of cadmium or zinc (35). Perhaps the most intriguing potential role for selenoprotein P comes from a recent study in which it was shown to copurify with a neuronal survival promoting activity in serum. As heavy metals are associated with toxicity to neurons and with neurodegenerative diseases, including Alzheimer’s and Parkinson’s disease (36–38), the proposed functions of heavy metal detoxification and promoting neuronal survival may in fact be linked. Studies to investigate this possible linkage are currently in progress.

Although the levels of recombinant selenoprotein P produced in HEK cells clearly do not constitute overexpression, we believe this system affords several advantages over purification of endogenously expressed protein from cell culture media or animal serum. These include the availability of media from mock- or vector-transfected cells to serve as a more appropriate negative control for functional assays than would be available with endogenously expressed protein and the ability to use mutagenesis to assess specific regions of the protein required for particular functions. For example, are 10 selenocysteine codons required for a particular activity or would, for example, 6 be sufficient? Are the conserved histidine stretches involved in the reported neuronal survival activity in the heavy metal binding activity or in both? What is the relationship between the two SECIS elements and 10 UGA codons? Do particular elements serve particular codons, as has been speculated by Hill et al. (2) and Himeno et al. (27)? Does translation through the multiple codons involve a processive mechanism or a reprogramming of the ribosomes, perhaps at initiation, such that all codons are then translated efficiently by that ribosome, as speculated by Kollmus et al. (39) and by Atkins et al. (40)?

The ability to express, purify, and immunologically detect recombinant selenoprotein P provides important avenues for pursuing studies of the functions of this intriguing protein, as well as more general questions on the mechanism of selenocysteine incorporation and, specifically, of multiple selenocysteine residues in a single polypeptide.

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