Phospholipid Hydroperoxide Glutathione Peroxidase Is the 18-kDa Selenoprotein Expressed in Human Tumor Cell Lines*

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Human tumor cell lines cultured in ^{75}Se-containing media demonstrate four major ^{75}Se-labeled cellular proteins (57, 22, 18, and 12 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Among these selenoproteins, an enzymatic activity is known only for the 22-kDa protein, since this protein has been identified as the monomer of glutathione peroxidase. However, all tested cell lines also contained a peroxidase activity with phospholipid hydroperoxides that is completely accounted for by the other selenoenzyme, phospholipid hydroperoxide glutathione peroxidase (PHGPX) (Ursini, F., Maiorino, M., and Gregolin, C. (1985) Biochim. Biophys. Acta 839, 62–70). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of ^{75}Se-labeled proteins separated by gel permeation chromatography supported the identification of PHGPX as the monomeric protein matching the 18 kDa band. This paper is the first report on the identification of PHGPX in human cells.

Phospholipid hydroperoxide glutathione peroxidase (PHGPX)[1] is a selenium-dependent peroxidase first described in 1982 by Ursini et al. (1). This enzyme specifically reduces hydroperoxide derivatives of intact phospholipids (2). Moreover, it has recently been shown that PHGPX also accounts for the enzymatic reduction of hydroperoxides of cholesterol and cholesterol ester in membranes and low density lipoproteins (3, 4). This selenoenzyme is distinct from cellular glutathione peroxidase (GPX) (EC 1.11.1.9, which is hereafter referred to as "classical," on the basis of molecular weight, amino acid composition, and substrate specificity (2, 5). The two peroxidases are expressed to different degrees in rat organs (6), and PHGPX is far more resistant to selenium deficiency than is GPX in mice (7).

Kinetic data suggest that GPX and PHGPX play different roles in hydroperoxide metabolism in cells, since in the aqueous phase the reaction of GPX with peroxides is favored over that with PHGPX (5, 8). PHGPX, on the other hand, specifically reduces hydroperoxides in membranes (2, 3) that are totally resistant to GPX (9). PHGPX is present in the cytosol of different tissues (6). Therefore, although PHGPX is catalytically active with membrane substrates and some activity has been actually detected in membranes (particularly in testis),[2] in the majority of examined tissues this enzyme is mostly cytosolic. The present study concerns the cytosolic form of the enzyme. In this paper, the presence of PHGPX in seven cell lines derived from human tumors is reported. The enzyme was identified as distinct among both the cellular peroxidase activities and the major ^{75}Se-labeled proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—The cell lines HL-60 and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD). Hep G2 was kindly provided by Dr. B. Knowles (Wistar Institute, Philadelphia) and also obtained from the American Type Culture Collection. K562 and the doxorubicin-resistant subline K562 ADR[R] were kindly provided by S. Yanovich (Virginia Commonwealth University, Richmond, VA). The doxorubicin-resistant sublines, HL-60 ADR[R] and MCF-7 ADR[R], were provided by Drs. K. Ross (Stanford University, Palo Alto, CA) and K. Cowen (NIH), respectively. MCF-7 ADR[R] is the subline resistant to doxorubicin at 10 μM. The attached cell lines, Hep G2, MCF-7, and MCF-7 ADR[R], were grown in minimal essential medium with Earle's salts, L-glutamine, nonessential amino acids, and 5% heat-inactivated fetal bovine serum (GIBCO). The suspension cell lines K562 and K562 ADR[R] were grown in RPMI 1640 and 5% heat-inactivated fetal bovine sera; HL-60 and HL-60 ADR[R] were grown in RPMI 1640 plus 10% heat-inactivated fetal bovine sera. All lines were grown in RPMI 1640 plus 10% heat-inactivated fetal bovine sera. All lines were maintained at 37 °C in a humidified atmosphere of air and 5% CO₂.

The Hep G2 cell line was also grown in serum-free media as described in Ref. 10.

Metabolic Labeling—[^{75}Se]Selenious acid (13–20 mCi/mg, Du Pont-New England Nuclear) was used as the sole selenium supplement when it was used to label cells. The reagent was found to be completely interchangeable with sodium selenite (Sigma). Cells were labeled by serial passage in media with fetal bovine serum and ^{75}Se for approximately 4–6 doubling times. Longer labeling periods (up to 35 days) did not alter the composition of selenopeptides observed by autoradiography of the SDS-PAGE-fractionated samples nor increase the GPX and PHGPX activity levels. Shorter selenite-labeling and induction times resulted in less detectable GPX and PHGPX activity.

Preparation of Cytosols—Cell lines were rinsed free of media twice with ice-cold phosphate-buffered saline (Dulbecco's, no calcium, pH 7.4, GIBCO). Attached cell lines were detached by scraping in phosphate-buffered saline. Cell lines were then rinsed once in 10 mM Hepes (pH 7.4), 3 mM MgCl₂, 4 mM EGTA plus a 1,000-fold dilution of a protease inhibitor stock solution composed of 17 mg/ml phenylmethylsulfonyl fluoride, 17 mg/ml benzamide, 5 mg/ml leupeptin, and 5 mg/ml pepstatin A in dimethyl sulfoxide (all protease inhibitors

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[1] The abbreviations used are: PHGPX, phospholipid hydroperoxide glutathione peroxidase; GPX, glutathione peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] A. Roveri, M. Maiorino, and F. Ursini, unpublished results.

from Sigma). The cell pellets were recovered by centrifugation and resuspended in 5 volumes (v/v) of the same solution. The cells were disrupted by 40 strokes of a hand-held, low clearance, glass homogenizer (Kontes, Vineland, NJ). The homogenate was centrifuged twice at 6,000 × g for 10 min to remove coarse particulate matter and nuclei and once at 100,000 × g for 1 h to remove membrane and small organelles. The samples were stored at −80 °C until use, which was routinely less than 1 week.

Analysis of Selenoproteins—Protein content of the samples was determined by the bicinchoninic acid method (BCA reagents, Pierce Chemical Co.) with bovine serum albumin as the standard.

Samples were gel-fractionated using SDS-PAGE composed of 12% acrylamide and 0.37% bisacrylamide as described by Laemmli (11). Electrophoresis was performed at a constant current of 4–6 mA/slab overnight. Gels were stained and fixed in a glacial acetic acid, methanol, water (10:40:50) containing 0.1% (w/v) Coomassie Brilliant Blue R for 30 min and then destained in the fix solution for 2 h and dried for autoradiography. The film was XAR-5 (Kodak), and the exposure was at −80 °C with two Du Pont Cronex Plus HE screens. The molecular mass standards were bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (14.2 kDa).

Gel permeation chromatography was performed on a 45 × 1.5 cm Sephadex G-200 column at 4 °C in 0.1 M Tris-HCl (pH 7.2), 1 mM glutathione, 5 mM EDTA, 5 mM EGTA. The void volume was determined by chromatography of blue dextran (2,000 kDa, Sigma), and the standards bovine serum albumin, carbonic anhydrase, and ribonuclease A were used to calibrate molecular mass.

Glutathione S-transferase Activity Measurement—Glutathione *S*-transferase activity was performed with 1-chloro-2,4-dinitrobenzene as the electrophilic substrate at 27 °C as described by Warholm *et al.* (12).

PHGPX Activity Measurement—PHGPX activity was measured by recording at 340 nm the specific NADPH oxidation in the presence of GSH, GSSG reductase, and phosphatidylcholine hydroperoxides, as previously described (2, 6, 13). Briefly, the reaction mixture contained, in 2.5 ml, 0.1 M Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% (v/v) Triton X-100, 0.2 mM NADPH, 3 mM GSH, 1 unit of GSH reductase, and the sample containing enzyme activity. After recording the baseline rate of nonspecific NADPH oxidation (which was subtracted from the activity), the reaction was started by adding 15–20 μM phosphatidylcholine hydroperoxides in a small volume of methanol (30 μl, maximum). The nonenzymatic oxidation of GSH in the presence of phosphatidylcholine hydroperoxides (*i.e.* the blank in the absence of enzyme activity) was negligible in these experimental conditions. Phosphatidylcholine hydroperoxides were prepared as described (13).

GPX Activity Measurement—GPX activity was measured using a test similar to that used for PHGPX activity except that GSH was 2 mM and Triton X-100 was omitted. The substrate was 0.25 mM H_2O_2. Since in the presence of H_2O_2 the nonenzymatic GSH oxidation was significant, this background was evaluated separately and subtracted.

Due to the different specific backgrounds in the presence of H_2O_2 or phosphatidylcholine hydroperoxides, the enzymatic activity detection threshold in our samples was 1.5–2 and 7–10 nmol/min/mg of protein for PHGPX and GPX, respectively.

RESULTS

Activities of PHGPX in breast cancer MCF-7, erythroleukemic K562, promyelocytic HL-60, and their doxorubicin-resistant variants ADR[R], as well as the hepatoma Hep G2 cell line, are reported in Fig. 1 (*panel A*). This figure also reports the specific peroxidase activity with H_2O_2 (that is accounted for by GPX) in the same cell lines (*panel B*).

Although quite low in MCF-7 ADR[R], PHGPX activity was present at relatively constant levels in all the examined lines. On the other hand, GPX was barely detectable in K562, K562 ADR[R], and MCF-7 cytosol, indicating that the two selenium peroxidases are independently expressed. As indicated in the figure, the activities of both GPX and PHGPX depend on the availability of selenium. Omission of selenite supplementation to basal medium with fetal bovine serum caused a 2–10-fold decrease of PHGPX and a 3–9-fold decrease of GPX activity. Furthermore, omission of selenite supplementation to Hep G2 cells cultured in a complete serum-free media resulted in
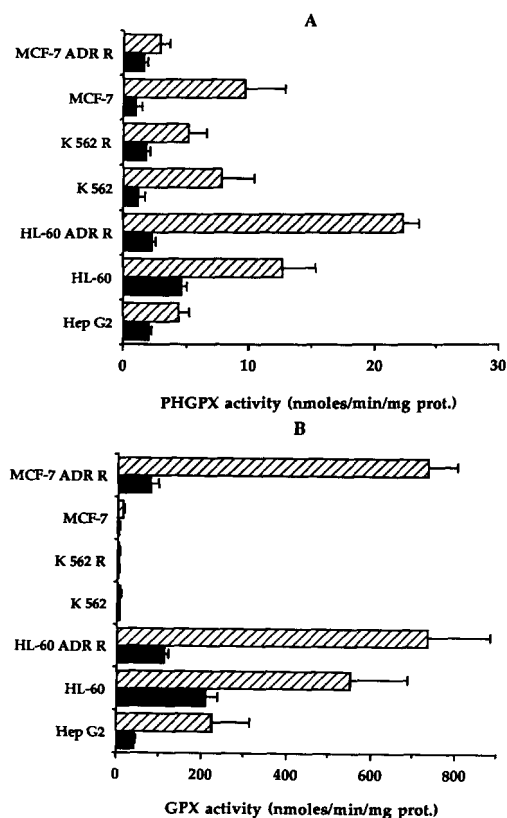

FIG. 1. **Effect of selenium supplementation on PHGPX and GPX activity of human tumor cell lines cytosol.** Cells were grown in a medium supplemented for 8 days (*hatched bars*) or not supplemented (*solid bars*) with 100 nM sodium selenite or [75Se] selenious acid. PHGPX (*panel A*) and GPX activity (*panel B*) were measured in cytosol as described under "Experimental Procedures."

an almost complete disappearance of GPX and PHGPX (data not shown). The MCF-7 ADR[R] variant behaved differently from the other cells since PHGPX activity was resistant to induction by selenite, whereas GPX activity was fully induced.

In order to identify the protein that accounted for PHGPX activity among the selenoproteins identified by radioactive selenium, we have used two of the above cell lines: the Hep G2 line, expressing both GPX and PHGPX, and the MCF-7 line, expressing PHGPX at the same level, but GPX at a very low level. Glutathione transferase activity with 1-chloro-2,4-dinitrobenzene was also measured in these cells, since a peroxidase activity with organic hydroperoxides was reported for some glutathione transferase isoenzymes (14–18). Glutathione transferase specific activity was 118 ± 10.6 and 14.8 ± 9.6 nmol/min/mg of protein in Hep G2 and MCF-7, respectively.

Cells were grown in a medium containing 100 nM [75Se] selenious acid. Fig. 2 reports the pattern of 75Se-labeled proteins of the whole cytosol of the two cell lines. As indicated, four major selenoproteins are detected under these conditions, with the estimated molecular masses of 57, 22, 18, and 12 kDa. Actually, the 22 kDa spot appears as a doublet with a component at 23 kDa. The selenoprotein distribution pattern is similar in the two cell lines; however, as expected because of the lack of GPX activity in the MCF-7 cytosol, the doublet at 22–23 kDa was not detected in these cells.

The cytosol of these 75Se metabolically labeled cells was chromatographed on a Sephadex G-200 column, and measurements of radioactivity and enzymatic activities on the eluted fractions were carried out as well as SDS-PAGE and autoradiography (Figs. 3–5). The chromatography allowed the
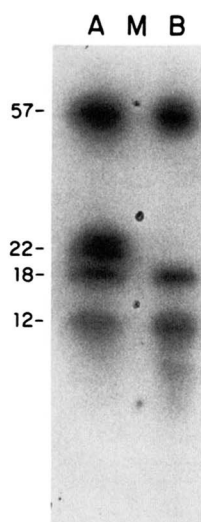
FIG. 2. **SDS-PAGE of selenoproteins in Hep G2 and MCF-7 cytosol after metabolic labeling with** [75]**Se.** Hep G2 (*A*) and MCF-7 (*B*) were metabolically labeled with ^{75}Se as described under "Experimental Procedures," and 52 µg of Hep G2 protein and 36 µg of MCF-7 protein (equal counts/min) of the cytosolic fraction were loaded in the wells. *Lane M* contained nonradiolabeled molecular mass standards that were marked on the autoradiograph in black marker.

separation of ^{75}Se into four major peaks referred to as I, II, III, and IV in both cell lines. One single peak of peroxidase activity with H_2O_2, one single peak of peroxidase activity with phosphatidylcholine hydroperoxides (Fig. 3, *panels A* and *B*), and one single peak of transferase activity with 1-chloro-2,4-dinitrobenzene (not reported) were detected in Hep G2 cells. Only the peroxidase activity with phosphatidylcholine hydroperoxides was observed in MCF-7 cells (Fig. 3, *panel D*) except for traces of transferase activity. Peak I was detected as a shoulder, just after the void volume of the Sephadex G-200 column, suggesting the presence of a high molecular weight selenoprotein. Only a 12-kDa selenoprotein was detected in these fractions by SDS-PAGE (Figs. 4 and 5). No peroxidase activities were detected in this fraction.

From both of the examined cell lines, peak II was eluted in a volume centered around 100 kDa, containing the bulk of the ^{75}Se activity. When Hep G2 cytosolic fractions were estimated on SDS-PAGE and autoradiography (Fig. 4), peak II was found to contain two distinct spots, at 57 and 22–23 kDa. On the other hand, only the 57-kDa selenoprotein was detected by SDS-PAGE in the corresponding cytosolic fractions from MCF-7 cells (Fig. 5). GPX activity was positively detected in the peak II fraction only in Hep G2 but not, as expected, in the corresponding MCF-7 fractions. Since peak II proteins were in the range of 100 kDa, these results indicate that the 57-kDa selenoprotein detected on SDS-PAGE is the monomer of a presumably dimeric protein and that the 22–23-kDa selenoproteins are different forms of the subunit of the "classical" tetrameric GPX (19).

Peak III was eluted on Sephadex G-200 in a volume centered around a molecular mass of 18 kDa (Fig. 3). From both examined cells, analysis on SDS-PAGE and autoradiography (Figs. 4 and 5) identified a single 18-kDa selenoprotein. Peak III contained all the peroxidase activity with phosphatidylcholine hydroperoxides present in the cytosol. The correspondence of molecular mass obtained by gel permeation and SDS-PAGE, as well as the activity on phosphatidylcholine hydroperoxides, indicates that peak III contains PHGPX, which is a monomeric enzyme. The small peak IV (Fig. 3),

which does not contain any selenoprotein, could possibly be ascribed to small selenium-containing peptides or nonprotein-bound selenium.

The peak of glutathione transferase activity matched neither peroxidase activity with H_2O_2 nor with phosphatidylcholine hydroperoxides (Fig. 3).

DISCUSSION

All of human tumor cell lines tested contained PHGPX activity, whereas a low activity was found in doxorubicin-resistant breast cancer cells (MCF-7 ADR[R]). Furthermore, in these cells the activity was scarcely increased by selenium supplementation, indicating that the low expression is not related to the low availability of this micronutrient.

The existence of a microsomal glutathione transferase that exhibits a specific peroxidase activity for dilinoleoyl phosphatidyl choline hydroperoxides, although at a specific activity 200-fold lower than PHGPX, has recently been reported (15). The activity of this transferase could, therefore, at least theoretically, bias PHGPX measurements if cytosol is contaminated with microsomes. However, while this membrane-bound transferase has been reported to require *N*-ethylmaleimide for full activation (15), no activation of peroxidase activity on phosphatidylcholine hydroperoxides has been observed in homogenates of different organs by such a treatment (7). Some other soluble glutathione transferase isoenzymes have been reported to catalyze the reduction of organic hydroperoxides at the expense of GSH (14, 16). In this reaction, GSH is oxidized to GSOH (17, 18), which can react with other thiols, giving rise to disulfides. Thus, the net reaction mimics that of selenium peroxidases even though the enzyme involved is not an oxidoreductase (20). This cytosolic "non-selenium-dependent glutathione peroxidase activity" has been reported with substrates such as cumene and free fatty acid hydroperoxides (14). There is no evidence that these enzymes could act on intact phospholipid hydroperoxides. Moreover, as indicated in Fig. 3, PHGPX activity was distinct and separated from glutathione transferase activity by gel permeation chromatography. Furthermore, the observed peroxidase activity with phosphatidylcholine hydroperoxides was dependent upon selenium supplementation (Fig. 1) and matched only the 18-kDa ^{75}Se-labeled band in SDS-PAGE (Figs. 4 and 5).

The trace activity obtained using H_2O_2 as substrate in K562, K562 ADR[R], and MCF-7 cells can actually be ascribed to PHGPX, since this activity is not associated with the expression of GPX protein by the criteria of ^{75}Se labeling (see Fig. 3 for MCF-7) and immunoprecipitation (not shown), and PHGPX has been shown to be catalytically competent for H_2O_2 reduction, although at a slow rate (8).

By metabolic labeling of cells with radioactive selenium, from 6–13 selenoproteins have been previously identified (23, 24). For none of these, other than GPX, has an enzymatic activity thus far been reported. The identification of PHGPX with one of these selenoproteins will help bridge the gap between the reported existence of a selenoprotein (with unknown enzymatic activity) and its identification as a specific enzyme. The lower number of selenoproteins detected in our study in comparison with previous reports (23, 24) could be due to intrinsic human tumor cell differences, but different labeling conditions, as well as different selenium status (24) before radioactive selenium supplementation, cannot be excluded. Nevertheless, since a common selenoproteins pattern can be extrapolated from different studies (23), we can draw conclusions concerning the pattern of the major selenoproteins that are compatible with both past and present work, in relation to the identification of one of them with PHGPX.
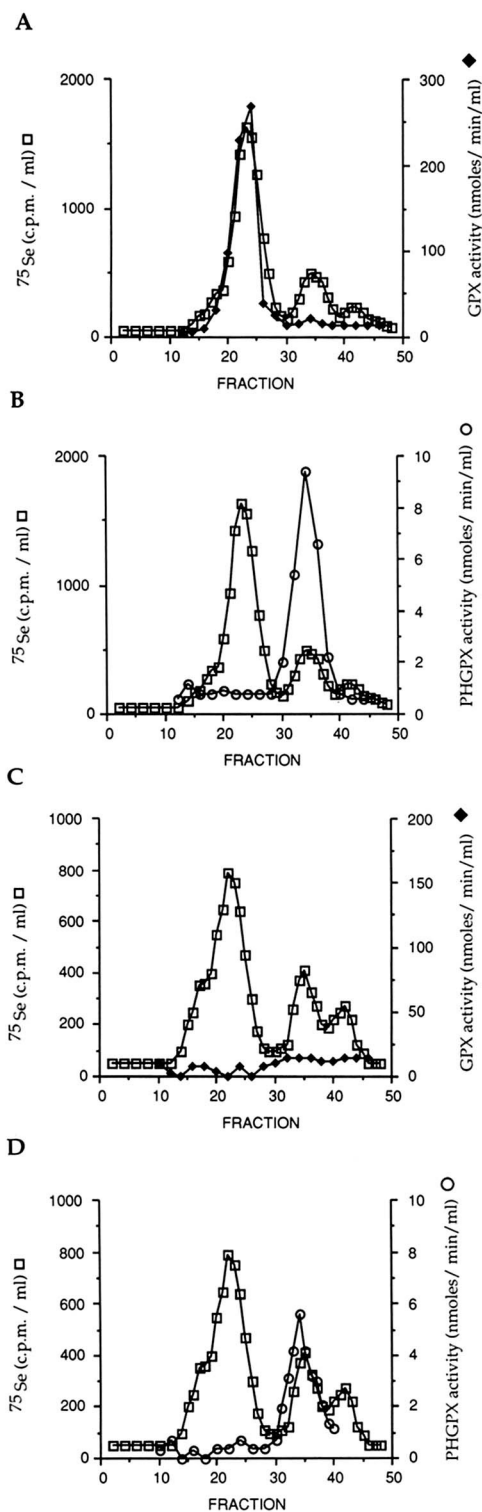
A

B

C

D

FIG. 3. **Sephadex G-200 column chromatography.** Hep G2 cell line and MCF-7 were metabolically labeled with ^{75}Se as described. Hep G2 cytosol (~7.3 mg of protein) (*panels A* and *B*) and MCF-7 cytosol (~5 mg of protein) (*panels C* and *D*) were chromatographed on a Sephadex G-200 column. The column was eluted with 0.1 M Tris-HCl buffer (pH 7.2), 5 mM EDTA, 5 mM EGTA, 1 mM GSH at a flow rate of 0.2 ml/min at 4 °C. Fractions of 1.95 ml were collected and analyzed for ^{75}Se and for GPX, PHGPX, and glutathione transferase activity. To calibrate the column, molecular weight standards were chromatographed alongside. *Panels A* and *C* report the chromatographic elution of radioactivity overlaid on the elution patterns for GPX. *Panels B* and *D* report the chromatographic elution of radioactivity overlaid on the elution patterns for PHGPX. The major ^{75}Se peaks were identified as follows: from fraction 10 to 18 (I), from
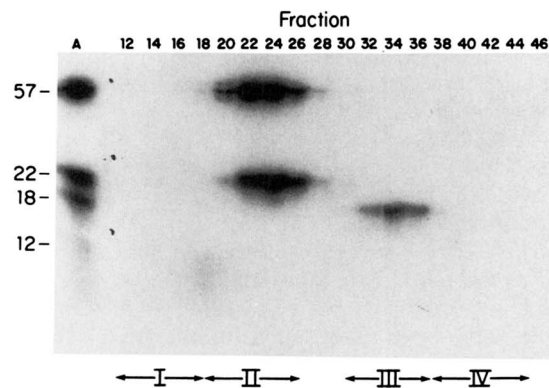

FIG. 4. **Electrophoretic pattern of Hep G2 cytosol seleno-proteins separated by Sephadex G-200 chromatography.** Whole cytosol (52 μg of protein) (*lane A*) and 0.075 ml of each chromatographic fraction from the same experiment as reported in Fig. 3 (*panels A* and *B*) were analyzed on SDS-PAGE with autoradiography to detect the ^{75}Se-labeled proteins as described under "Experimental Procedures." Molecular weight standards were chromatographed alongside.

FIG. 5. **Electrophoretic pattern of MCF-7 cytosol selenoproteins separated by Sephadex G-200 chromatography.** Whole cytosol (36 μg of proteins) (*lane A*) and 0.075 ml of each chromatographic fraction from the same experiment as reported in Fig. 3 (*panels C* and *D*) were analyzed by SDS-PAGE and autoradiography to detect the ^{75}Se-labeled proteins as described under "Experimental Procedures." Molecular weight standards were chromatographed alongside.
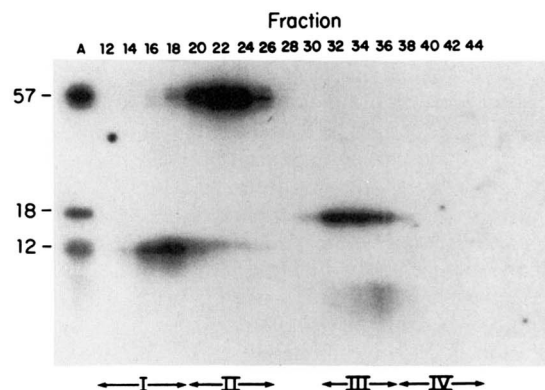
The ~57 kDa Selenoprotein—This protein fits our peak II of radioactive selenium, and its presence was reported in most of the tissue homogenates from ^{75}Se-treated animals (23–26). In some cases, this band was surrounded by two minor bands, whose molecular masses are a little higher and a little lower than 57 kDa. Notably, in our human tumor cell lines, the 57 kDa band appeared as a triplet on SDS-PAGE (see Figs. 2, 4, and 5).

No major selenoproteins are reported between these bands and the subunit of GPX. The present work confirms this evidence.

The "Classical" Tetrameric GPX—This enzyme also eluted in our peak II. Under our conditions on SDS-PAGE, the monomer appears as a doublet with a molecular mass of 22–23 kDa. This 23-kDa selenoprotein was detected in Hep G2 and Hep 3B (not shown) cell lines, both of liver origin. The 23-kDa protein cross-reacts to some degree with anti-human erythrocyte GPX IgG, but not with anti-human plasma GPX

fraction 19 to 27 (II), from fraction 31 to 37 (III), and from fraction 38 to 45 (IV). Glutathione transferase activity was eluted in a few fractions centered on fraction 28.

IgG.[3] GPX variants in human cell lines that migrate with higher apparent molecular masses due to nonconservative amino acid substitutions may account for the presence of the 23-kDa selenoprotein (27).[4]

The estimated molecular weight of the monomer of GPX by radioactivity after ^{75}Se labeling in tissues has been reported to be 26 kDa by Danielson and Medina (23) and 23.7 ± 7 kDa by Behne *et al.* (24).

PHGPX—This enzyme fits peak III of radioactive selenium in our G-200 chromatography. The 18-kDa selenoprotein eluted in peak III has been identified as PHGPX in the present study. Based upon similarities between PHGPX distribution in mammal tissues (6) and selenoprotein pattern in tissues and cells (23, 24), we propose that PHGPX is identical with the 22-kDa selenoprotein described by Danielson and Medina (23) and the 19.7-kDa protein reported by Behne *et al.* (24). The discrepancies in molecular mass exhibited by SDS-PAGE among different authors are small and within the range of experimental error by SDS-PAGE analysis. On the other hand, the PHGPX molecular mass reported here is slightly lower than that reported previously (2). Elucidation of PHGPX primary structure will provide the true molecular mass.

Other Selenoproteins—Under our conditions, only a 12-kDa selenoprotein was additionally found on SDS-PAGE. As this was found in the high molecular weight peak on gel permeation (peak I), this probably was a small selenoprotein subunit of a much larger multisubunit protein or a lipid-soluble protein that elutes near the void in association with lipid aggregates.

The identification of PHGPX in human cells helps to clarify one aspect of the biological role of selenium, specifically in relation to the antioxidant system where PHGPX activity accounts for the synergistic effect between selenium and vitamin E (28). Nevertheless, as membrane-bound lipid hydroperoxides have recently been suggested to have physiological roles (29), the identification of this enzyme in human tissue could have important consequences in understanding how these hydroperoxides are regulated.

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