Mitochondrial Phospholipid Hydroperoxide Glutathione Peroxidase Plays a Major Role in Preventing Oxidative Injury to Cells*

(Received for publication, June 8, 1998, and in revised form, September 10, 1998)

Masayoshi Araï, Hirotaka Imai, Tomoko Koumura, Madoka Yoshida, Kazuo Emoto, Masato Umeda, Nobuyoshi Chiba, and Yasuhiro Nakagawa‡

From the §School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, the §Department of Inflammation Research, Tokyo Metropolitan Institute of Medical Science (Rinshoken), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, and the ¶Japan Energy Corporation, 3-17-35 Nitto-Minami, Toda-shi, Saitama 335, Japan

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is synthesized as a long form (L-form; 23 kDa) and a short form (S-form; 20 kDa). The L-form contains a leader sequence that is required for transport to mitochondria, whereas the S-form lacks the leader sequence. A construct encoding the leader sequence of PHGPx tagged with green fluorescent protein was used to transfect RBL-2H3 cells, and the fusion protein was transported to mitochondria. The L-form of PHGPx was identified as the mitochondrial form of PHGPx and the S-form as the non-mitochondrial form of PHGPx since preferential enrichment of mitochondria for PHGPx was detected in M15 cells that overexpressed the L-form of PHGPx, whereas no similar enrichment was detected in L9 cells that overexpressed the S-form. Cell death caused by mitochondrial injury due to potassium cyanide (KCN) or rotenone (chemical hypoxia) was considerably suppressed in the M15 cells, whereas the L9 cells and control RBL-2H3 cells (S1 cells, transfected with the vector alone) succumbed to the cytotoxic effects of KCN. Flow cytometric analysis showed that mitochondrial PHGPx suppressed the generation of hydroperoxide, the loss of mitochondrial membrane potential, and the loss of plasma membrane integrity that are induced by KCN. Mitochondrial PHGPx might prevent changes in mitochondrial functions and cell death by reducing intracellular hydroperoxides. Mitochondrial PHGPx failed to protect M15 cells from mitochondrial injury by carbonyl cyanide m-chlorophenylhydrazone, which directly reduces membrane potential without the generation of hydroperoxides. M15 cells were more resistant than L9 cells to cell death caused by direct damage to mitochondria and to extracellular oxidative stress. L9 cells were more resistant to tert-butylhydroperoxide than S1 cells, whereas resistance to t-butylhydroperoxide was even more pronounced in M15 cells than in L9 cells. These results suggest that mitochondria might be a target for intracellular and extracellular oxidative stress and that mitochondrial PHGPx, as distinct form non-mitochondrial PHGPx, might play a primary role in protecting cells from oxidative stress.

Mitochondria are a major physiological source of reactive oxygen species (ROS), which can be generated during mitochondrial respiration. Superoxide radicals, formed by minor side reactions of the mitochondrial electron transport chain or by an NADH-independent enzyme, can be converted to H2O2 and to the powerful oxidant, the hydroxyl radical (2). Thus, mitochondria are continually exposed to ROS that cause peroxidation of membrane lipids, cleavage of mitochondrial DNA, and impairment of ATP generation, with resultant irreversible damage to mitochondria. Mitochondrial dysfunction might contribute to the pathogenesis of various human neurodegenerative disorders, such as Parkinson’s, Alzheimer’s, and Huntington’s diseases, amyotrophic lateral sclerosis, stroke, epilepsy, aging, and the AIDS dementia complex (3–5). However, ROS don’t have exclusively toxic effects; low levels of ROS generated in mitochondria can act as signaling molecules under physiological conditions. ROS produced in mitochondria can activate transcription factors, such as NF-κB and AP-1 (6), and can function as signals in apoptosis that is induced by TNF-α (7), ceramide (8), and chemical hypoxia (9).

The production of ROS in mitochondria is strictly regulated by mitochondrial antioxidant enzymes that include phospholipid hydroperoxide glutathione peroxidase (PHGPx), classical glutathione peroxidase (cGPx), and Mn-superoxide dismutase (Mn-SOD). The importance of antioxidant enzymes in mitochondria is indicated by the fact that knock-out mice without a gene for Mn-SOD suffer from catastrophic effects (10). By contrast, knock-out mice without a gene for cGPx are quite vigorous. Some of these tissues remain very resistant to oxidative stress even though GPx is the only antioxidant enzyme that is known to reduce the H2O2 produced by Mn-SOD in mitochondria since mitochondria in most mammalian cells lack catalase activity (11). Two types of GPx, namely cGPx and PHGPx, are located in mitochondria. PHGPx is the only known intracellular antioxidant enzyme that can directly reduce peroxidized phospholipids (12) and cholesterol (13) in membranes. Therefore, PHGPx that can reduce H2O2, rather than cGPx, is thought to contribute to the enzymatic defenses against oxidative damage to mitochondria (14). However, the PHGPx in mitochondria has not been fully characterized.

We previously cloned a cDNA for PHGPx from the rat (15, 17, 22). The abbreviations used are: ROS, reactive oxygen species; BSO, buthionine sulfoximine; CCCP, carbonyl cyanide m-chlorophenylhydrazone; cGPx, cytosolic glutathione peroxidase; DCFH-DA, 5,6-carboxy-2′,7′-dichlorofluorescin diacetate; GFP, green fluorescent protein; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PI, propidium io- diode; RBL, rat basophil leukemia cells; Rh123, rhodamine 123; t-BuOOH, tert-butylhydroperoxide; TNF-α, tumor necrosis factor-α; SOD, superoxide dismutase; BSA, bovine serum albumin.

* This work was supported in part by Special Coordination Funds for Promoting Science and Technology, and by Grants-in-aid 10672052 and 10780389 from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan. Fax: 81-3-344-4943; E-mail: nakagaway@pharm.kitasato-u.ac.jp.

‡ The abbreviations used are: ROS, reactive oxygen species; BSO, buthionine sulfoximine; CCCP, carbonyl cyanide m-chlorophenylhydrazone; cGPx, cytosolic glutathione peroxidase; DCFH-DA, 5,6-carboxy-2′,7′-dichlorofluorescein diacetate; GFP, green fluorescent protein; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PI, propidium iodide; RBL, rat basophil leukemia cells; Rh123, rhodamine 123; t-BuOOH, tert-butylhydroperoxide; TNF-α, tumor necrosis factor-α; SOD, superoxide dismutase; BSA, bovine serum albumin.
and demonstrated that a short 20-kDa (S-form) and a long 23-kDa (L-form) form of PHGPx were translated from the cDNA, which included two potential sites for the initiation of translation in vitro (16). We showed that the L-form included a leader sequence and was selectively imported into the mitochondria of rat liver by an import system in vitro (16).

Stable transformants of rat basophile leukemia 2H3 (RBL-2H3) cells, in which the S-form of PHGPx was overexpressed, were resistant to the cell death caused by a radical initiator or oxidized lipids (17). The S-form of PHGPx markedly inhibited the production of leukotrienes by 5-lipoxygenase by preventing production of intracellular hydroperoxides around the nucleus (18).

In the present study, RBL-2H3 cells that overexpressed the L-form of PHGPx were established and compared with those that overexpressed the S-form in an attempt to estimate the functional roles of the two types of PHGPx in protection against intracellular and extracellular oxidative stress. The L-form of PHGPx was more effective than the S-form in preventing cell death that was caused by ROS generated in mitochondria and by exogenously added hydroperoxides.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Antibodies against PHGPx and cGPx were prepared as described previously (17). Monoclonal antibodies against histone H1 and 12S1-protein A (2.60-3.70 Tbg/ml) were purchased from Cosmobio Co. Ltd. (Tokyo, Japan) and ICN Biochemicals Inc. (Irvine, CA), respectively. Rhodamine 123 (Rh123), 5,6-carboxy-2'7'-dihalo-fluorescein diacetate (DCFH-DA), and cis-parinaric acid were obtained from Funakoshi Co. Ltd. (Tokyo, Japan). Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) and tri-n-butyl phosphate were obtained from Wako Co. Ltd. (Tokyo, Japan). Propidium iodide (PI) and monoclonal antibodies against cytochrome oxidase subunit IV was obtained from Molecular Probes (Eugene, Oregon), KGN, rotenone, CCF, oligomycin, and BSO were purchased from Sigma.

**Construction of Plasmids—**A BamHI fragment of pRHGPx4 (16) was subcloned into pSRα, as the expression vector, to construct pSRα-L-form PHGPx that encoded the L-form of PHGPx (19). S-probe and L-probe were made from pRHGPx4 by polymerase chain reactions for construction of S-GFP and L-GFP. The primers for construction of the S-probe, in which the cDNA encoded the 42 amino acids from the first residue of the S-form of PHGPx, were 5'-ACATAAAGCTTGTCGGACCATGTTGCA-3' and 5'-ATAGTGATCCGCAGGTGGGATGAT-3'. The primers for the construction of the L-probe, in which the cDNA encoded the 32 amino acids from the first residue of the L-form of PHGPx, were 5'-ATTTAAGCTTCCGCGCAGTGAGACC-3' and 5'-ATTAGCTACCGCGGAGTACACAGTGT-3'. The BamHI and KpnI fragments of S-probe and L-probe were inserted between the BamHI and KpnI sites of the GFP expression vector (pcMX-SAPY1145 F) that had been constructed by Ogawa et al. (20).

**Cell Culture and Transfection—**We used the previously established control line of cells (S1 cells) and L9 cells that overexpressed the L-form (non-mitochondrial) of PHGPx (17). M15 cells, which overexpressed the L-form (mitochondrial) of PHGPx, were established by the transfection of cDNA into RBL-2H3 cells with pSRα-L-form PHGPx and pSV2neo by electroporation, as described previously (17). A suspension of RBL-2H3 cells (1 x 10^7 cells/0.25 ml) was transferred to an electroporation cuvette (0.4-cm gap) of 0.4% Triton X-100 for 10 min at 4 °C, and each cDNA encoding an amino acid sequence of PHGPx (mitochondrial) was introduced into the nucleus (18). The nuclear, mitochondrial, and microsomal fractions were solubilized in 200 μl of 0.4% Triton X-100 in PBS for 2 h at 4 °C, and each supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatants were supplemented into 400 μl each of PBS and subjected to immunoprecipitation with antibodies against PHGPx and cGPx, as described previously (17). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) and analyzed by autoradiography. Total levels of PHGPx and cGPx were calculated from results of scanning densitometry after autoradiography with a Bio Imaging Analyzer (BAS2000; Fuji Film, Tokyo).

**Activities of PHGPx and cGPx—**Activities of PHGPx and cGPx were measured after the fractionation of cells by the dual mitochondrial and non-mitochondrial immunoprecipitation. The nuclear, mitochondrial, and microsomal fractions were solubilized in 200 μl of 0.4% Triton X-100 in PBS for 2 h at 4 °C, and each supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatants were supplemented into 400 μl each of PBS and subjected to immunoprecipitation with antibodies against PHGPx and cGPx, as described previously (17). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) and analyzed by autoradiography. Total levels of PHGPx and cGPx were calculated from results of scanning densitometry after autoradiography with a Bio Imaging Analyzer (BAS2000; Fuji Film, Tokyo).

**Protection of Cells from Oxidative Injury by PHGPx—**Stable transformants of rat basophile leukemia 2H3 (RBL-2H3) cells, which overexpressed the mitochondrial PHGPx were isolated. Control cells and non-mitochondrial PHGPx overexpressing cells including S1 and L9 cells has been determined (18). The purity of each subcellular fraction of M15 cells was determined according to our previous paper in which the the purity of each organelle of control cells and non-mitochondrial PHGPx overexpressing cells including S1 and L9 cells has been determined (18). The purity of each subfractionation of M15 cells was the same as those in S1 and L9 cells. Cytochrome c oxidase was distributed in nuclear (7.9%), mitochondrial (75.6%), microsomal (3.5%), and cytosolic (12.9%) fractions of M15 cells. NADPH-cytochrome c reductase activities were found in nuclear (5.9%), mitochondrial (16.7%), microsomal (68.3%), and cytosolic (9.1%) fractions of M15 cells.

The nuclear, mitochondrial, and microsomal fractions were solubilized in 200 μl of 0.4% Triton X-100 in PBS for 2 h at 4 °C, and each supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatants were supplemented into 400 μl each of PBS and subjected to immunoprecipitation with antibodies against PHGPx and cGPx, as described previously (17). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) under non-reducing conditions. Gels were stained, dried, and subjected to autoradiography. Total levels of PHGPx and cGPx were calculated from results of scanning densitometry after autoradiography with a Bio Imaging Analyzer (BAS2000; Fuji Film, Tokyo).

**Distribution of PHGPx in Cultured Cells—**Activities of PHGPx and cGPx were measured after the fractionation of cells by the dual mitochondrial and non-mitochondrial immunoprecipitation. The nuclear, mitochondrial, and microsomal fractions were solubilized in 200 μl of 0.4% Triton X-100 in PBS for 2 h at 4 °C, and each supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatants were supplemented into 400 μl each of PBS and subjected to immunoprecipitation with antibodies against PHGPx and cGPx, as described previously (17). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) under non-reducing conditions. Gels were stained, dried, and subjected to autoradiography. Total levels of PHGPx and cGPx were calculated from results of scanning densitometry after autoradiography with a Bio Imaging Analyzer (BAS2000; Fuji Film, Tokyo).

**GFP and mitochondria were simultaneously detected in the same cells by the double staining with GFP fluorescence and a monoclonal antibody of Cy3-conjugated anti-cytochrome c oxidase subunit IV that was a specific probe for the mitochondrial staining (27). Fixed cells were incubated with pH 7.4 saline (18). The specificity of SOD was measured in terms of the percentage inhibition of the formation of superoxide by the xanthine-xanthine oxidase system (26). Mn-SOD activity was measured in the presence of 5 mM KCN, and Cu,Zn-SOD activity was calculated by subtraction of the activity of Mn-SOD from the total SOD activity.
in the same cells was monitored and photographed with an appropriate filter pack.

Cell Viability—S1, L9, and M15 cells were plated at 0.5 × 10^5 cells/well in flat-bottomed 96-well culture plates and cultured for 24 h. Individual transformants were exposed to indicated doses of KCN, rotenone, CCCP, oligomycin, or t-BuOOH for appropriate periods. The LDH release assay was used for the determination of the cell viability, as described elsewhere (17). In one series of experiments, cells were incubated for 12 h prior to exposure to KCN with 0.5 mM buthionine sulfoxamine (BSO) for depletion of GSH.

Flow Cytometric Analysis—Changes in the integrity of plasma membrane and in the mitochondrial membrane potential were examined by monitoring staining with propidium iodide (PI) and Rh123, respectively. After treatment with KCN, cells were stained with PI (5 mg/ml) and Rh123 (1 mg/ml) for 10 min. We also used an oxidation-sensitive fluorescent probe, 5,6-carboxy-2',7'-dichlorofluorescein-diacetate (DCFH-DA), to assess levels of intracellular peroxides, as follows. Cells were washed with PBS and incubated with 2.5 μM DCFH-DA in PBS for 15 min. DCFH-loaded cells were incubated with or without 25 mM KCN for the times indicated. The intensity fluorescence from PI, Rh123, and dichlorofluorescein (DCF) in cells was analyzed with a flow cytometer (EPICS® Elite Flow cytometer; Coulter, Hialeah, FL).

Analysis of Cellular Levels of ATP—Cellular levels of ATP were determined by the luciferin-luciferase method using a kit from Sigma (29).

Fluorescence Measurements of Lipid Peroxidation—Cells (1 × 10^6 cells) were loaded with 20 mM cis-parinaric acid for 1.5 h at 37 °C and then washed with PBS. The loaded cells were treated with 25 mM KCN for the times indicated. After incubation, total lipids were extracted as described by Bligh and Dyer (30). Fluorescence of total lipids was monitored with a spectrofluorometric detector (RF-550; Shimazu Co. Ltd., Japan) with excitation at 393 nm and emission at 416 nm.

Fluorescence Measurements of GSH in Mitochondria and Cytosol—Amounts of GSH in mitochondria and cytosol were measured according to the previous paper with a slight modification (28). In brief, S1, L9, and M15 cells (each 2 × 10^7 cells) were fractionated into cytosol and mitochondria. Cytosol and mitochondria dispersed with the sonication were precipitated by trichloroacetic acid at a final concentration of 5%. After centrifugation at 10,000 × g for 10 min, GSH in the supernatant was converted to fluorescent derivative. The reaction was started by the addition of 0.5 ml of 0.02% ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) in 0.25 M borate buffer, pH 10.5, which contained 5 mM EDTA and 1% tri-n-butyl phosphine.

The reaction mixture kept at 60 °C for 30 min, and the reaction was terminated by the addition of 50 μl of 4 × HCl, and fluorescent thiol derivatives were separated by reversed-phase high pressure liquid chromatography (TSK-gel ODS; TOSOH Co. Ltd., Japan). The mobile phase was 0.1 mM citrate buffer, pH 4.0, tetrahydrofuran/acetonitrile (94.8:0.2:5). The fluorescent thiol derivatives were monitored with emission at 516 nm and excitation at 384 nm during elution at a flow rate of 1.0 ml/min.

Quantitation of Proteins—Concentrations of protein were determined with the BCA protein assay reagent (Pierce), with bovine serum albumin (BSA) as the standard.

Expression of Results—All data from assays in which the number of replicates was three or more are expressed as mean values ± S.D.

RESULTS

Sorting of Green Fluorescent Protein Tagged with the Leader Sequence of PHGPx into the Mitochondria of RBL-2H3 Cells—The L-form of PHGPx contains a leader sequence, but the S-form does not (Fig. 1). Chimeric proteins that included green fluorescent protein (GFP) were expressed in RBL-2H3 cells in order to determine whether the leader sequence of the L-form could serve to target GFP to the mitochondria of living cells. One fusion protein consisted of GFP with the leader sequence of 32 amino acids from the first residue of L-form (L-GFP). The other was a fusion protein of GFP with 42 amino acids from the first residue of S-form (S-GFP). Expression vectors containing cDNA that encoded GFP, L-GFP, or S-GFP were used to transfect RBL-2H3 cells by electroporation and then the intracellular localization of fluorescence due to GFP was monitored with a fluorescence microscope 24 h later (Fig. 2). Fluorescence was diffusely distributed in cells that expressed S-GFP or GFP (Fig. 2, A and B). By contrast, discrete regions with strong fluorescence were observed in cells that expressed L-GFP (Fig. 2C). GFP and mitochondria in the L-GFP-transfected cells were simultaneously visualized by the double staining with GFP fluorescence and a monoclonal antibody of Cy3-conjugated anti-cytochrome c oxidase subunit IV (Fig. 2, C and D). The profile of fluorescence due to L-GFP was identical to that of mitochondrial cytochrome c oxidase. Efficient import of GFP with the leader sequence of L-form into mitochondria indicates that the leader sequence at the amino terminus of mitochondrial PHGPx is the signal for targeting to mitochondria.

Subcellular Localization of PHGPx and cGPx in RBL-2H3 Cells That Overexpressed the L-form and the S-form of PHGPx—RBL-2H3 cells were transfected by electroporation with cDNAs that encoded the L-form and the S-form of PHGPx (Fig. 1A). Two types of transformant that stably expressed substantial levels of PHGPx were isolated after appropriate selection. M15 cells strongly expressed the L-form of PHGPx with the leader sequence and L9 cells expressed the S-form of PHGPx. The control line of cells (S1) had been transfected with the expression vector without an insert. The three kinds of transformants were labeled with [75Se]sodium selenite for 4...
days for determination of the amounts of PHGPx and cGPx (Table I). The total amounts of PHGPx in L9 and M15 cells were 4 and 3.5 times higher than that in S1 cells, respectively. No significant differences in total respective amounts of cGPx, Cu,Zn-SOD, and Mn-SOD were detected among L9, M15, and S1 cells.

Fig. 3 shows the subcellular distribution of 75Se-labeled PHGPx and cGPx in L9, M15, and S1 cells. In S1 cells, PHGPx was more concentrated in the mitochondria than in the cytosolic and microsomal fractions (Fig. 3, A and C). Levels of PHGPx were significantly elevated in the cytosolic, microsomal, and nuclear fractions in L9 cells. The amount of PHGPx in the mitochondrial fraction from L9 cells was 4 times higher than that from S1 cells, but the amounts of PHGPx in the mitochondrial fractions from L9 and S1 cells were similar. The amount of PHGPx in the mitochondrial fraction from M15 cells was twice that from S1 cells. The overexpression of L-form PHGPx caused the enhancement of the activity of PHGPx in mitochondria. Specific activity of PHGPx in mitochondria of M15 cells was 272 ± 12 pmol/min/mg, whereas specific activities in S1 and L9 were 156 ± 18 and 156 ± 37 pmol/min/mg, respectively. The activities of cytosolic PHGPx in S1, L9, and M15 cells were 4.0 ± 1.6, 16 ± 1.9, and 8.2 ± 1.7 pmol/min/mg, respectively. These results show that the leader sequence of the L-form of PHGPx is the targeting signal for transport to mitochondria, whereas the S-form PHGPx is widely distributed in various organelles. The L-form of PHGPx can be considered to be the mitochondrial PHGPx and the S-form to be the non-mitochondrial PHGPx.

In three types of cells, cGPx was localized exclusively in the cytosolic fraction (Fig. 3, B and D). In S1 cells, the amount of cGPx in the mitochondria was lower than that of PHGPx. No significant changes of cGPx activities in cytosol and mitochondria were found by the overexpression of PHGPx (data not shown).

Resistance of Transformant Cells to Oxidative Damage to Mitochondria—The sensitivity of mitochondria in M15, L9, and S1 cells to injury was evaluated by exposing cells to KCN, an inhibitor of the respiratory chain (chemical hypoxia) (Fig. 4). The viability of S1 cells and L9 cells decreased rapidly in a time- and dose-dependent manner, and only about 20% of cells remained viable after exposure to 25 mM KCN for 6 h (Fig. 4, A and B). By contrast, M15 cells were much more resistant to cell death caused by KCN. The LD50 of KCN for M15 cells was approximately 30 mM, whereas LD50 for L9 and S1 cells was 20 mM. These results indicated that mitochondrial damage by KCN could be prevented to some extent by the overexpression of PHGPx. However, overexpression of non-mitochondrial PHGPx did not have such a protective effect.

Effects of KCN on the viability of S1, L9, and M15 cells that had been depleted of GSH were examined to estimate whether or not the resistance to KCN of M15 cells had resulted from overexpression of mitochondrial PHGPx activity. Buthionine
sulfoximine (BSO), an inhibitor of the synthesis of glutathione, inhibits the activity of glutathione-dependent peroxidases, such as cGPx and PHGPx, by lowering the level of glutathione in cytosol and mitochondria of cells. Amounts of glutathione in cytosol and mitochondria of S1 cells were 35.6 ± 2.8 and 18.2 ± 1.6 nmol/mg protein, respectively. No significant changes of glutathione content were observed in the PHGPx-overexpressing cells. The levels of cellular glutathione were markedly reduced by the treatment with BSO. Amounts of glutathione in cytosol and mitochondria of S1 cells reduced to 3.39 ± 2.8 and 2.80 ± 1.32 nmol/mg protein by the treatment of BSO, respectively. In BSO-treated M15 cells, amounts of glutathione in cytosol and mitochondria were 3.38 ± 1.33 and 2.75 ± 1.69 nmol/mg protein, respectively. Decrease in the level of glutathione by BSO was also found in L9 cells at the same extent as that in M15 cells (data not shown). When M15 cells pretreated with BSO were exposed to KCN, the cells lost their resistance to KCN toxicity (Fig. 4C). These results confirm that resistance of M15 cells to KCN is due to the overexpression of PHGPx in mitochondria.

### Table I
The levels of selenium-labeled PHGPx and cGPx and of Mn-SOD and Cu,Zn-SOD in S1, L9, and M15 cells

| Strain                     | 75Se-PHGPx  | 75Se-cGPx  | Mn-SOD  | Cu,Zn-SOD |
|----------------------------|-------------|-------------|---------|-----------|
| Control line of cells      | S1          | L9          | M15     |
| Non-mitochondrial PHGPx-overexpressing cells | 4.62 ± 0.13 × 10^4 | 18.0 ± 0.7 × 10^4 | 16.1 ± 0.6 × 10^4 |
| Mitochondrial PHGPx-overexpressing cells | 21.4 ± 0.8 × 10^4 | 20.6 ± 0.9 × 10^4 | 20.8 ± 0.8 × 10^4 |
|                           | 0.21 ± 0.02 | 0.18 ± 0.03 | 0.24 ± 0.01 |
|                           | 9.66 ± 0.61 | 10.70 ± 2.73 | 10.00 ± 1.52 |

---

**Fig. 3.** Subcellular localization of PHGPx and cGPx in PHGPx-overexpressing RBL-2H3 cells. S1 cells (white bars), L9 cells (gray bars), and M15 cells (black bars) were incubated with 75Se (sodium selenite; 0.14 mCi/ml) for 4 days. 75Se-labeled cells were fractionated by differential centrifugation into a nuclear (Nu), a mitochondrial fraction (Mit), a microsomal fraction (Mic), and a cytosolic fraction (Cyt). Distributions of PHGPx and cGPx were determined by immunoprecipitation with antibodies against PHGPx (A and C) and against cGPx (B and D). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) with subsequent autoradiography (C and D). Radioactivity (in relative units) was quantified with a Bio-Imaging Analyzer.

**Fig. 4.** Effects of KCN on the viability of PHGPx-overexpressing cells. S1 cells (closed circles), L9 cells (closed triangles), and M15 cells (closed squares) were exposed to 25 mM KCN for the times indicated (A). Individual cultures were exposed to the indicated concentrations of KCN for 6 h (B). Individual cultures were treated for 12 h with 0.5 mM BSO to deplete cells of intracellular GSH and were then exposed to the indicated concentrations of KCN for 6 h. After incubation, cell viability was determined from the extent of release of LDH. Viability was expressed as a percentage relative to the total LDH in the cells. The total LDH in cells was determined after lysis of cells with 0.2% Triton X-100. Data are the means ± S.D. of results from four replicates in each case.
respectively. As shown in Fig. 5A, rotenone had a strong toxic effect on L9 and S1 cells, whereas M15 cells were more resistant to cell death caused by rotenone. By contrast, the sensitivity of M15 to mitochondrial injury due to oligomycin or to CCCP was the same as that of S1 and L9 cells (Fig. 5, B and C). These results indicate that mitochondrial PHGPx contributes to the protection of cells from cell death due to inhibitors of the respiratory chain but not from death due to the direct effects on membrane potential and reduction on the production of ATP. Non-mitochondrial PHGPx failed to protect cells from cell death that was caused by any impairment of mitochondrial function.

Inhibition of the Cyanide-induced Generation of Hydroperoxides by Mitochondrial PHGPx—The effects of KCN on intracellular levels of hydroperoxides in the individual transformants were determined by flow cytometry using the oxidant-sensitive dye 2',7'-dichlorofluorescein (Fig. 6). In S1 cells, we detected the rapid generation of hydroperoxides within 30 min (Fig. 6A). Hydroperoxides were produced in KCN-treated L9 cells to the same extent and with the same time course as in S1 cells (Fig. 6B). However, the production of hydroperoxides was considerably suppressed in KCN-treated M15 cells as compared with KCN-treated S1 and L9 cells (Fig. 6C).

Suppression of the KCN-induced Peroxidation of Lipids by Mitochondrial PHGPx—We next examined peroxidation of lipids in KCN-treated transformants. cis-Parinaric acid, which is a naturally fluorescent polyunsaturated fatty acid, was used as a sensitive indicator of lipid peroxidation in cells. A reduction in the intensity of fluorescence of cis-parinaric acid is an indicator of lipid peroxidation (31). Analysis of the fluorescence of cis-parinaric acid indicated that the fluorescence decreased within as little as 1 h after the start of exposure of cells to KCN. Fluorescence from S1 and L9 cells was reduced to 50% of the original value after treatment with KCN for 1 h (Fig. 7). By contrast, no reduction in fluorescence from M15 cells was observed after 1 h, an indication that lipid peroxidation was suppressed in M15 cells as compared with L9 cells.

Protection from Disruption of Mitochondrial Functions by Mitochondrial PHGPx—To assess changes in mitochondrial membrane potential (Δψ) and in the integrity of plasma membranes in transformant cells, we performed flow cytometric analysis after double staining with rhodamine 123 (Rh123) and propidium iodide (PI) (Fig. 8). Rh123, a lipophilic cation, is selectively taken up by mitochondria, and uptake is directly proportional to mitochondrial Δψ. PI is imported into cells and binds to cellular DNA when the integrity of plasma membranes is lost. Cells that had not been treated with KCN were predominantly located in the PI-negative and high Δψ field (PI(−)Δψhigh) (Fig. 8, A, D, and G). New subsets of S1 cells appeared after treatment of S1 cells with KCN for 2 h; a large number of subsets was located in the PI(−)Δψlow field and a smaller subset was present in the PI(+)Δψlow field (Fig. 8B). Most S1 cells lost membrane integrity upon exposure to KCN for 4 h and moved to the PI(−)Δψlow field (Fig. 8C). Changes of the distribution of L9 cells upon treatment with KCN were expected to be identical to those of S1 cells. Indeed, L9 cells also shifted from the PI(−)Δψlow field to the PI(+)Δψlow field upon exposure to KCN (Fig. 8, E and F). By contrast, M15 cells retained their mitochondrial membrane potential and remained in the PI(−)Δψhigh field after the exposure to KCN for 2 h (Fig. 8H). Many M15 cells retained their mitochondrial membrane potential even after long term treatment with KCN (4 h) (Fig. 8I). These observations indicate that mitochondrial PHGPx protects cells by preventing loss of membrane potential and loss of membrane integrity, whereas non-mitochondrial PHGPx can't protect cells from the effects of KCN.

Rapid reductions in levels of cellular ATP were observed in S1 and L9 cells after 2 h of exposure to KCN, with further losses within the next 2 h (Table II). The level of ATP in M15 cells after 3 h of treatment with KCN was clearly much higher than in S1 and L9 cells. The decreases in levels of ATP corresponded closely to the loss of mitochondrial membrane potential in transformant cells upon treatment with KCN.

Protection from Disruption of Mitochondrial Functions Induced by t-BuOOH—Changes in mitochondrial functions induced by t-BuOOH were estimated by flow cytometric analysis (Fig. 10). Three subsets of S1 cells appeared after exposure to t-BuOOH for 1 h as follows: cells in the PI(−)Δψhigh field; cells in the PI(−)Δψmoderate field; and cells in the PI(+)Δψlow field. Thus, S1 cells lost mitochondrial membrane potential and also plasma membrane integrity, and they were located predominantly in the PI(−)Δψlow field after treatment with t-BuOOH (Fig. 10A). Moderate decomposition of plasma membranes and the moderate loss of mitochondrial potential were observed in L9 cells. More L9 cells remained in the PI(−)Δψmoderate field than S1 cells (Fig. 10B). The membrane potential of M15 cells was retained, and cells with a low membrane potential were
Protection of Cells from Oxidative Injury by PHGPx

Fig. 6. Flow cytometric analysis of intracellular hydroperoxides in PHGPx-overexpressing cells exposed to KCN. To assess levels of intracellular peroxides, flow cytometric analysis was performed with the fluorescent probe DCFH-DA. S1 cells (A), L9 cells (B), and M15 cells (C) were preincubated with DCFH-DA for 5 min at 37 °C and then incubated with 25 mM KCN (black areas) or without KCN (white areas) for 1 h. The intensity of fluorescence from DCFH of cells was quantified by flow cytometry and is plotted on a logarithmic scale, in arbitrary units, against the number of cells.

Fig. 7. Effects of KCN on the peroxidation of lipids in PHGPx-overexpressing cells. S1 cells (closed circles), L9 cells (closed triangles), and M15 cells (closed squares) were incubated with 20 μM cis-parinaric acid for 90 min at 37 °C. Then the cells were exposed to 25 mM KCN for the indicated times. After the incubation with KCN, lipids were extracted, and the fluorescence of total lipids was quantified with a spectrofluorometric detector. The intensity of fluorescence from KCN-treated cells is expressed as a percentage relative to the intensity of fluorescence from control cells. Data are means ± S.D. of triplicate results.

fewer than in the case of L9 cells (Fig. 10C). The population of M15 cells located in the PI(−)−Δψmoderate field was also smaller than in the case of L9 and S1 cells.

DISCUSSION

Some proteins in mitochondria are initially synthesized in the cytoplasm as larger precursors, and then they are imported into the mitochondria where proteolytic cleavage yields the mature forms. The leader sequence of the 23-kDa precursor to PHGPx is the signal for import of this protein into the mitochondria by using in vitro import system (16). In the present study, we examined the transport of PHGPx into the mitochondria of living cells. Green fluorescent protein (GFP) has proved useful as a probe in an attempt to visualize the translocation of proteins in living cells. However, few studies have used GFP to study the transport of proteins into mitochondria in cells. Yano et al. (32) successfully visualized the translocation of a chimeric protein that consisted of GFP and the presequence of ornithine transcarbamylase among the organelles of COS 7 cells. We constructed a plasmid that encoded GFP protein linked to the leader sequence of PHGPx (L-GFP) to investigate the role of the leader sequence. L-GFP was correctly targeted to the mitochondria, whereas S-GFP, which lacked the leader sequence, was not detected in mitochondria (Fig. 2). Thus, the leader sequence of 23-kDa PHGPx (L-form) appears to be required not only as an import signal but also as a targeting signal. There are four known isoforms of glutathione peroxidases as follows: cytosolic GPx (cGPx), PHGPx, plasma GPx, and gastrointestinal GPx (33). PHGPx is unique among these isoforms in having a leader sequence for transport to mitochondria. cGPx is located in mitochondria but is not translated with a signal peptide.

The leader sequence of PHGPx is located between two different sites for initiation of translation of the cDNA for PHGPx. Pushpa-Rekha et al. (34) demonstrated that the gene for PHGPx has alternative transcription sites and that these sites result in two populations of mRNAs for PHGPx. They found that one mRNA had an upstream AUG codon that was primarily utilized in rat testis and was translated to yield the mitochondrial PHGPx (L-form). The other mRNA lacked the upstream AUG codon, and it encoded non-mitochondrial PHGPx (S-form), which was synthesized predominantly in somatic cells. In RBL-2H3 cells, the upstream initiation site appears to be used predominantly, with resultant transcription of mRNA for mitochondrial PHGPx, since the amount of PHGPx in mitochondria was higher than that in other organelles (Fig. 3). The mechanism for regulation of transcription from the two initiation sites in a single gene for PHGPx remains to be resolved.

A considerable amount of PHGPx was found in mitochondria when RBL-2H3 cells were transfected with a plasmid encoding the L-form of PHGPx (M15 cells). Levels of PHGPx were also elevated in cytosolic, microsomal, and nuclear fractions of M15 cells as compared with S1 cells (control transfected cells). Two possibilities can be considered to explain the increased amounts of PHGPx in organelles other than mitochondria in M15 cells. One possibility is that mRNA for the S-form of PHGPx is transcribed from the downstream initiation site, as well as from the upstream site, when the L-form of PHGPx is overexpressed. Alternatively, the signal peptide of PHGPx might be partially removed by proteolysis before import into mitochondria can be completed. The latter possibility can be rejected since the fusion protein that consisted of GFP with the signal peptide was efficiently transported into mitochondria (Fig. 2).

The overexpression of the mitochondrial type of PHGPx protected RBL-2H3 cells from cell death due to mitochondrial oxidative stress that resulted from exposure of cells to KCN and rotenone (chemical hypoxia). Neither S1 nor L9 cells were resistant to the cytotoxicity of KCN. Resistance of M15 cells was eliminated when the activity of PHGPx in these cells was inhibited by depletion of cytosolic and mitochondrial glutathione.

Some proteins in mitochondria are initially synthesized in the cytoplasm as larger precursors, and then they are imported into the mitochondria where proteolytic cleavage yields the mature forms. The leader sequence of the 23-kDa precursor to PHGPx is the signal for import of this protein into the mitochondria by using in vitro import system (16). In the present study, we examined the transport of PHGPx into the mitochondria of living cells. Green fluorescent protein (GFP) has proved useful as a probe in an attempt to visualize the translocation of proteins in living cells. However, few studies have used GFP to study the transport of proteins into mitochondria in cells. Yano et al. (32) successfully visualized the translocation of a chimeric protein that consisted of GFP and the presequence of ornithine transcarbamylase among the organelles of COS 7 cells. We constructed a plasmid that encoded GFP protein linked to the leader sequence of PHGPx (L-GFP) to investigate the role of the leader sequence. L-GFP was correctly targeted to the mitochondria, whereas S-GFP, which lacked the leader sequence, was not detected in mitochondria (Fig. 2). Thus, the leader sequence of 23-kDa PHGPx (L-form) appears to be required not only as an import signal but also as a targeting signal. There are four known isoforms of glutathione peroxidases as follows: cytosolic GPx (cGPx), PHGPx, plasma GPx, and gastrointestinal GPx (33). PHGPx is unique among these isoforms in having a leader sequence for transport to mitochondria. cGPx is located in mitochondria but is not translated with a signal peptide.

The leader sequence of PHGPx is located between two different sites for initiation of translation of the cDNA for PHGPx. Pushpa-Rekha et al. (34) demonstrated that the gene for PHGPx has alternative transcription sites and that these sites result in two populations of mRNAs for PHGPx. They found that one mRNA had an upstream AUG codon that was primarily utilized in rat testis and was translated to yield the mitochondrial PHGPx (L-form). The other mRNA lacked the upstream AUG codon, and it encoded non-mitochondrial PHGPx (S-form), which was synthesized predominantly in somatic cells. In RBL-2H3 cells, the upstream initiation site appears to be used predominantly, with resultant transcription of mRNA for mitochondrial PHGPx, since the amount of PHGPx in mitochondria was higher than that in other organelles (Fig. 3). The mechanism for regulation of transcription from the two initiation sites in a single gene for PHGPx remains to be resolved.

A considerable amount of PHGPx was found in mitochondria when RBL-2H3 cells were transfected with a plasmid encoding the L-form of PHGPx (M15 cells). Levels of PHGPx were also elevated in cytosolic, microsomal, and nuclear fractions of M15 cells as compared with S1 cells (control transfected cells). Two possibilities can be considered to explain the increased amounts of PHGPx in organelles other than mitochondria in M15 cells. One possibility is that mRNA for the S-form of PHGPx is transcribed from the downstream initiation site, as well as from the upstream site, when the L-form of PHGPx is overexpressed. Alternatively, the signal peptide of PHGPx might be partially removed by proteolysis before import into mitochondria can be completed. The latter possibility can be rejected since the fusion protein that consisted of GFP with the signal peptide was efficiently transported into mitochondria (Fig. 2).

The overexpression of the mitochondrial type of PHGPx protected RBL-2H3 cells from cell death due to mitochondrial oxidative stress that resulted from exposure of cells to KCN and rotenone (chemical hypoxia). Neither S1 nor L9 cells were resistant to the cytotoxicity of KCN. Resistance of M15 cells was eliminated when the activity of PHGPx in these cells was inhibited by depletion of cytosolic and mitochondrial glutathione.
one with buthionine sulfoximine (BSO). Thus, overexpression of mitochondrial PHGPx clearly contributed to protection from mitochondrial damage.

We next investigated how mitochondrial PHGPx interferes with the toxicity of KCN, an inhibitor of complex IV of the mitochondrial respiratory chain. KCN causes various types of damage to mammalian cells, inducing the rapid generation of ROS (35), the reduction of mitochondrial Δψ (36), a decrease in cellular levels of ATP (36), and lipid peroxidation (37), for example. These phenomena developed in KCN-treated RBL-2H3 cells in a time-dependent manner. Flow cytometric analysis revealed that KCN induced the rapid generation of hydroperoxides in S1 cells within 30 min (Fig. 6A–C), L9 cells (D–F), and M15 cells (G–I) were double-stained with Rh123 and PI after the incubation with 25 mM KCN for 0 h (A, D, and G), 2 h (B, E, and H) and 4 h (C, F, and I). The intensity of fluorescence from PI was plotted against that from Rh123. Similar results were obtained in three independent experiments.

Mitochondrial PHGPx hindered the generation of hydroperoxides, which was an early feature of cell damage. Mitochondrial PHGPx failed to prevent cell death in response to CCCP or oligomycin, both of which directly reduced the membrane potential and the level of cellular ATP without the production of hydroperoxides. Thus, mitochondrial PHGPx appeared to maintain the functions of mitochondria by reduction of intracellular hydroperoxides generated as a result of damage to the mitochondrial respiratory machinery.

PHGPx in mitochondria effectively reduced the H₂O₂ generated in mitochondria that had been damaged by exposure to KCN since the extent of lipid peroxidation caused by H₂O₂ was significantly reduced in KCN-treated M15 cells. In general, PHGPx reduces H₂O₂ less effectively than cGPx. The present results suggest that PHGPx in mitochondria might limit local increases in concentrations of H₂O₂. By contrast, PHGPx in the cytosol might reduce H₂O₂ that is dispersed in the cytosol much less efficiently.

M15 and L9 cells exhibited different levels of sensitivity to extracellular oxidative damage (Fig. 9). M15 cells were more sensitive to extracellular oxidative damage than L9 cells.
Protection of Cells from Oxidative Injury by PHGPx

resistant to the cytotoxic effects of t-BuOOH than L9 cells even though the total activity of intracellular PHGPx was similar in both lines of transformed cells. These results suggest that mitochondria might be a primary target for t-BuOOH. Mitochondrial PHGPx prevents cell death by protecting the mitochondrial machinery from t-BuOOH (Fig. 10). Mitochondrial PHGPx not only prevents direct injury of mitochondria by KCN, but it also reduces the cytotoxicity of exogenously added t-BuOOH by protecting mitochondrial functions. Levels of ROS in mitochondria increase substantially in pathological situations, such as ischemia reperfusion (38). Several factors have been reported that protect mitochondria against oxidative damage. Mitochondrial SOD (Mn-SOD) is induced by TNF-α, which initiates cell death through the elevation of levels of ROS in mitochondria (39). Induced Mn-SOD might protect cells from the toxic effects of TNF-α (40). Heat shock proteins are also induced to protect cells from stresses that include ROS. Overexpression of heat shock protein 70 results in resistance to the cytotoxicity of TNF-α. Polla et al. (41) demonstrated that heat shock protein 70 prevented changes in mitochondrial membrane potential by H$_2$O$_2$, and they suggested that mitochondria might be protective targets for protective effects against the oxidative injury. Bcl-2, which is an anti-apoptosis protein, is located primarily on the outer membrane of mitochondria. Bcl-2 prevents cells from undergoing necrosis in response to inhibitors of the respiratory chain (chemical hypoxia) such as rotenone, antimycin A, and KCN (36) and from apoptosis in response to a variety of other stimuli (42, 43). Bcl-2 prevents cell death by inhibiting the loss of mitochondrial membrane potential (36) and by inhibiting the release of cytochrome c, an activator of caspases (44, 45), from the mitochondria to the cytosol. Exogenous H$_2$O$_2$ is a potent inducer of the liberation of cytochrome c from damaged mitochondria, and Bcl-2 effectively prevents the toxic effects of exogenous H$_2$O$_2$ that lead to cell death (43). These earlier results suggest that the generation of ROS in the mitochondria might be a key step in the initiation of cell death and that mitochondria are well placed to be sensors of oxidation damage to cells (46). PHGPx is localized predominantly at contact sites between the outer and inner membranes of mitochondria in the rat testes (47). The reduction of levels of ROS in mitochondria by PHGPx might also be associated with the defenses of the cell against apoptosis that is mediated by damages to mitochondria.

Recent reports indicate that ROS play an important role as mediators or modulators in cellular signaling pathways, such as the Ras signaling pathway (48), the mitogen-activated protein kinase pathway (49), and the activation of nuclear factor κB (NFκB) (50). ROS generated in mitochondria might be responsible for the activation of NFκB (51) and of Sterile 20 (Ste20), which is likely to be an oxidant-stress-responsive kinase-1 (SOK-1) (52). Brigelius-Flohe et al. (53) found that the activation of NFκB by interleukin-1 was inhibited in ECV304 cells that overexpressed PHGPx. The present study also suggests that mitochondrial PHGPx might participate in the regulation of signal transduction pathways that are triggered by ROS in mitochondria.

Acknowledgments—We thank Akiko Tondo, Aya Teshirogi, and Atsuko Suito for their expert technical assistance. We also thank Dr. Kazuhiko Umesono and Dr. Junichiro Inoue for the kind gift of the GFP expression vector, and we thank members of the Kitasato Institute for help in the flow cytometric analysis.

REFERENCES
1. Guarneri, C., Muscar, C., and Caldarella, C. M. (1992) in Free Radicals and Aging (Emeriti, I., and Chance, B., eds) pp. 73–77, Birkauser Verlag, Basel
2. Nohl, H. (1987) FEMS Lett. 214, 269–273
3. Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10771–10778
4. Leistinen, P., and Bataille, N. (1994) Biomed. Pharmacother. 48, 199–214
5. Tristschler, H. J., Packer, L., and Medorri, R. (1994) Biochem. Mol. Biol. Int. 34, 169–181
6. Rao, G. N., Glasgow, W. C., Eling, T. E., and Runge, M. S. (1996) J. Biol. Chem. 271, 27760–27764
7. Donato, N. J., and Perret, M. (1998) J. Biol. Chem. 273, 5067–5072
8. Quillet-Mary, A., Jaffrezou, J.-P., Mansat, V., Bordier, C., Naval, J., and Laurent, G. (1997) J. Biol. Chem. 272, 21388–21395
9. Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995) J. Biochem. 119, 1255–1259
10. Li, Y., Huang, T. T., Carleon, R. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., and Epstein, C. J. (1995) Nat. Genet. 11, 376–381
11. Esoworthy, R. S., Ho, Y. S., and Chu, P. F. (1997) Arch. Biochem. Biophys. 340, 59–63
12. Ursini, F., Maiorino, M., and Gregolin, C. (1985) Biochim. Biophys. Acta 839, 62–72
13. Thomas, J. P., Maiorino, R., Gregolin, C., and Ursini, F. (1990) J. Biol. Chem. 265, 454–461
14. Flores, H. (1989) in Glutathione: Chemical, Biochemical, and Medical Aspects (Dolphins, D., Poulson, R., and Arramovic, O., eds) pp. 643–731, John Wiley & Sons, Inc., New York
15. Imai, H., Sumi, D., Hanamoto, A., Arai, M., Sugiyama, A., Chiba, N., Kuchino, Y., and Nakagawa, Y. (1995) J. Biochem. (Tokyo) 118, 1061–1067
16. Arai, M., Imai, H., Sumi, D., Imayama, T., Nakamura, T., Imanaka, T., Chiba, N., and Nakagawa, Y. (1996) Biochem. Biophys. Res. Commun. 225, 432–439
17. Imai, H., Sumi, D., Sakamoto, H., Hanamoto, A., Arai, M., Chiba, N., and Nakagawa, Y. (1996) Biochem. Biophys. Res. Commun. 222, 432–438
18. Imai, H., Narashima, K., Arai, M., Sakamoto, H., Chiba, N., and Nakagawa, Y. (1996) J. Biol. Chem. 271, 1960–1967
19. Takebe, Y., Seki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472
20. Ogasawa, H., Inouye, S., Tsuchi, F., Yasuda, K., and Umesono, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11899–11903
21. Southen, P. J., and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327–341
22. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1965) Biochem. J. 60, 694–617
23. Roderick, A. C., Michael, F. M., and Jan-Willem, T. (1995) Methods Enzymol. 260, 117–132
24. Imanaka, T., Shiina, Y., Takano, T., Hashimoto, T., and Osumi, T. (1996)
Protection of Cells from Oxidative Injury by PHGPx

25. Lawrence, J. J., and Daure, M. (1976) Biochemistry 15, 3301–3307
26. Crapo, J. D., McCord, J. M., and Fridovich, I. (1978) Methods Enzymol. 53, 382–383
27. Capaldi, R. A. (1990) Annu. Rev. Biochem. 59, 569–596
28. Imai, K., Toyo’oka, T., and Watanabe, Y. (1983) Anal. Biochem. 128, 471–473
29. Giorgi, S. D., and Michel, B. L. (1990) Anal. Biochem. 190, 304–308
30. Bligh, E. D., and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 911–918
31. McGuire, S. O., James-Kracke, M. R., Sun, G. Y., and Fritsche, K. L. (1997) Lipids 32, 219–226
32. Yano, M., Kanazawa, M., Terada, K., Namech, C., Yamaizumi, M., Hanson, B., Hosenraad, N., and Mori, M. (1997) J. Biol. Chem. 272, 8459–8465
33. Chu, F. F. (1994) Cytogenet. Cell Genet. 66, 96–98
34. Pushpa-Rekha, T. R., Burdsall, A. L., Oleksa, L. M., Chisolm, G. M., and Driscoll, D. M. (1995) J. Biol. Chem. 270, 26993–26999
35. Edward, M. M., Palur, G. G., Geran, P., and Gary, E. I. (1996) J. Neurochem. 67, 1039–1046
36. Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996) Oncogene 13, 21–29
37. Myers, K. M., Fiskum, G., Liu, Y., Simmens, S. J., Breidesen, D. E., and Murphy, A. N. (1995) J. Neurochem. 65, 2472–2440
38. Schonheit, K., Gille, L., and Nohl, H. (1995) Biochim. Biophys. Acta 1271, 335–342
39. Nishi, T., Richter, C., and Peterhans, E. (1993) Biochem. J. 289, 587–592
40. Weng, G. N., Elwell, J. H., Oberley, L. W., and Goeddel, D. V. (1989) Cell 58, 923–931
41. Polla, B. S., Kantengwa, S., Francois, D., Salviole, S., Franceschi, C., Marsac, C., and Cossart, Z. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6458–6463
42. Hocherhery, D. M., Ottiati, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 75, 241–251
43. Zhang, P., Liu, B., Kang, S. W., Seo, M. S., Rhee, S. G., and Obeid, L. M. (1997) J. Biol. Chem. 272, 30615–30618
44. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Brando, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) Science 275, 1129–1132
45. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
46. Wylee, A. (1997) Nature 388, 237–238
47. Godeas, C., Sandri, G., and Panfili, E. (1994) Biochim. Biophys. Acta 1191, 147–150
48. Lander, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995) J. Biol. Chem. 270, 21195–21198
49. Guyton, K. Z., Liu, Y., Gerolpe, M., Xu, Q., and Holbrook, N. J. (1995) J. Biol. Chem. 271, 4138–4142
50. Piette, J., Piret, B., Boninzi, G., Schoonbroodt, S., Merville, M. P., Legrand-Poels, S., and Bours, V. (1995) Biochim. Biophys. Acta 1278, 1237–1245
51. Garcia-Ruiz, C., Codd, A., Morales, A., Kaplowsky, N., and Fernandez-Checa, J. C. (1995) Mol. Pharmacol. 48, 825–834
52. Pombo, C. M., Tozijita, T., Kyrakiakos, J. M., Bonventre, J. V., and Force, T. (1997) J. Biol. Chem. 272, 29372–29379
53. Brigitte-Flohe, R., Friendrichs, B., Maurer, S., Schultz, M., and Streicher, R. (1997) Biochem. J. 15, 199–203