Oxidative protein folding occurs primarily in the mammalian endoplasmic reticulum, enabled by a diverse network comprising more than 20 members of the protein disulfide isomerase (PDI) family and more than five PDI oxidases. Although the canonical disulfide bond formation pathway involving Ero1α and PDI has been well-studied so far, the physiological roles of the newly identified PDI oxidases, glutathione peroxidase-7 (GPx7) and -8 (GPx8), are only poorly understood. We here demonstrated that human GPx7 has much higher reactivity with H2O2 and hence greater PDI oxidation activity than human GPx8. The high reactivity of GPx7 is due to the presence of a catalytic tetrad at the redox-active site, which stabilizes the sulfenylated species generated upon the reaction with H2O2. Although it was previously postulated that GPx7 catalysis involved a highly reactive peroxidatic cysteine that can be sulfenylated by H2O2, we revealed that a resolving cysteine instead regulates the PDI oxidation activity of GPx7. We also determined that GPx7 formed complexes preferentially with PDI and P5 in H2O2-treated cells. Altogether, these results suggest that human GPx7 functions as an H2O2-dependent PDI oxidase in cells, whereas PDI oxidation may not be the central physiological role of human GPx8.

Most secretory and cell-surface membrane proteins undergo oxidative protein folding in the endoplasmic reticulum (ER) to acquire native conformations via disulfide bond formation and isomerization. In the mammalian ER, the reactions are catalyzed by a variety of oxidoreductases that encompass more than 20 members of the protein disulfide isomerase (PDI) family (1–5) and more than 5 PDI oxidases including ER oxidoreductin-1α (Ero1α) (6–9) and peroxiredoxin-4 (Prx4) (10–12). Some PDI family enzymes also engage in disulfide bond reduction to promote protein folding (13), the ER-associated degradation of misfolded proteins (14–16), or the ER-to-cytosol retrograde translocation of a bacterial cholera toxin and nonenveloped viruses (17–19). PDI family members typically possess thioredoxin-like domains with a CXXC motif at the redox-active site, thereby exerting thiol-disulfide exchange reactions with substrate proteins (1, 3). Thus, the PDI family enzymes, in combination with their upstream oxidases or reductases, constitute a diverse thiol-mediated network to maintain the proteostasis in the ER (2, 3).

Ero1α and PDI play central role in the canonical pathway of disulfide bond formation at the cost of one molecular oxygen per disulfide bond, resulting in the generation of H2O2 as a byproduct (2, 6). Overproduction of H2O2 as a consequence of the Ero1α overwork causes oxidative stress in the ER, eventually leading to cell death. Therefore, the oxidative activity of Ero1α needs to be tightly controlled (20). In the mechanism of operation of mammalian Ero1α, four cysteine residues in the electron shuttle loop act also as components of a regulatory switch, which ensures strict regulation over Ero1α activity (8, 9, 20–23). An ER-resident peroxiredoxin, Prx4, metabolizes H2O2 and oxidizes PDI family enzymes upon return to the reduced state (24, 25). Our prior in vitro studies showed that ERp46 and P5, with their partner oxidase Prx4, are engaged in the rapid but promiscuous disulfide bond introduction in the initial phase of oxidative protein folding (10), whereas PDI, in concert with Ero1α, efficiently catalyzes the correction of non-native disulfide bonds and the subsequent selective formation of native disulfide bonds (26). Other oxidative pathways involving vitamin K epoxide reductase were also reported to potentially operate in the PDI reoxidation cycle (27, 28).

More recently, glutathione peroxidase-7 (GPx7) and -8 (GPx8) were identified as ER-resident PDI oxidases using H2O2 as a source of oxidative power (29–31). GPx family was historically named after its first identified member that catalyzes the reaction of peroxide with reduced glutathione (GSH) on its selenocysteine residue (32). Despite the absence of a GSH-binding motif and the substitution of the selenocysteine residue by Cys in GPx7 and GPx8, both the enzymes are capable of acting as peroxidases, which reduce H2O2 to water (29, 31, 33). GPx7-deficient cells were found to accumulate endogenous reactive oxygen species, lowering cellular viability (34, 35). Another study demonstrated that GPx7 plays a role in the alleviation of oxidative stress in breast cancer cells, esophageal cells, and adipose tissue, emphasizing the importance of GPx7 in the oxidative stress response (35–38). Both GPx7 and GPx8 were suggested to interact with Ero1α in vivo and possibly modulate the peroxide-generating activity of Ero1α by scavenging H2O2 in its proximity (29). Additionally, the Ero1α–GPx8
enzymes with H2O2 have not been assessed precisely. To compare their peroxidatic activities, we first mixed the fully reduced GPx7 or the luminal domain of GPx8 (1 μM each) with 10, 50, or 200 μM of H2O2, and the redox-state change of PDI was monitored by far-UV CD spectroscopy (Fig. 1). Consequently, GPx7 generated significant amounts of partially and fully oxidized PDI at early time points, whereas GPx8 hardly accelerated PDI oxidation compared with H2O2 alone. This result is in line with the previous observation that GPx7 was oxidized by H2O2 much more efficiently than GPx8 (Fig. 1). Of note, GPx7-mediated PDI oxidation was slower at 200 μM H2O2 than at 50 μM H2O2. This is likely explained by the greater generation of a hyperoxidized species of GPx7 species during the catalysis of PDI oxidation in the presence of higher concentrations of H2O2. In support of this notion, most of GPx7 was converted to a hyperoxidized species during the catalysis of PDI oxidation at 200 μM H2O2 (Fig. 2B). The generation of a hyperoxidized species of GPx7 was greater in the presence of reduced PDI (Fig. 2B versus Fig. 1, left, middle, and bottom panel). This observation suggests that the catalytic cycle significantly increased the hyperoxidized species via redox reactions between H2O2 and reduced GPx7 generated by oxidizing PDI every turnover.

A pKd value, which determines the degree of protonation at a given pH, represents a fundamental character of protein thiol groups (42, 43). To examine whether the pKd values of GPx7 and GPx8 can explain their different peroxidatic activities, we analyzed crystal structures of the enzymes (Protein Data Bank codes 2P3I for GPx7 and 3CYN for GPx8) using the program PROPKA3. The in silico analysis yielded the pKd values of peroxidatic cysteines (Cr) of GPx7 and GPx8 to be 9.3 and 11.5, respectively. Thus, Cr in GPx8 is predicted to have exceptionally low reactivity compared with Cr in GPx7 and free cysteine in solution (pKd of 8.3). Altogether, the results tempt us to suspect a functional role of GPx8 as a H2O2-scavenging peroxidase in the ER.

Biochemical characterizations of GPx7 and GPx8

Effective thiol-based peroxidases commonly contain a catalytic triad or tetrad including a highly reactive, Cp (40, 41). Indeed, GPx7 contains a catalytic tetrad consisting of Cys57, Trp142, Asn143, and Glu92 at the active site, where Glu92 is predicted to stabilize thiolated or sulfenylated Cys57 through a hydrogen bond upon reaction with H2O2 (40). Although GPx8 also conserves the first three residues, Cys59, Trp164, and Asn165, at the positions corresponding to Cys57, Trp142, and Asn143 in GPx7, respectively, we surmised that the replacement of Glu92 (in GPx7) to Ser112 (in GPx8) (Fig. 3, A and B) was a primary reason for much lower peroxidase activity of GPx8 than that of GPx7. In this context, the distance between the Sγ atom of GPx7 Cys57 and Oε1 of Glu92 is 3.1 Å, close enough to stabilize the thiolated GPx7 Cp via a hydrogen bond to the Oε1; meanwhile the Sγ atom of GPx8 Cys79 is separated from the Oε atom of Ser114 by 4.8 Å, far beyond a hydrogen-bonding distance (Fig. 3B).
To explore the importance of the CP-neighboring residues in the different peroxidatic activities of GPx7 and GPx8, we constructed swap mutants where Gln is mutated to Ser in GPx7 (GPx7 Q92S) and vice versa in the luminal domain of GPx8 (GPx8 S114Q). In the result, the GPx7 swap mutation greatly compromised the GPx7 reactivity with H$_2$O$_2$ (Fig. 3C, left panels), leading to the abolishment of PDI oxidation activity (Fig. 3D, left panel). Notably, GPx7 Q92S exhibited a similar rate in H$_2$O$_2$-dependent oxidation as GPx8, although the latter generated larger amount of hyperoxidized species than the former at both 10 and 50 $\mu$M H$_2$O$_2$ (Figs. 1 and 3C, left panel). It is also noteworthy that GPx8 S114Q greatly increased the GPx8 reactivity with H$_2$O$_2$, leading to more efficient PDI oxidation in the presence of 10 $\mu$M H$_2$O$_2$ (Fig. 3C and D, upper right panel). However, GPx8 S114Q only partially oxidized PDI at 50 $\mu$M H$_2$O$_2$ (Fig. 3D, lower right panel), likely because of the predominant formation of the hyperoxidized form as observed for GPx7 WT in the presence of 200 $\mu$M H$_2$O$_2$ and 10 $\mu$M reduced PDI (Fig. 2B, right panel). Even without reduced PDI, GPx8 S114Q indeed generated a considerable amount of hyperoxidized species upon reaction with 50 $\mu$M H$_2$O$_2$ (Fig. 3C, lower right panel). Collectively, we conclude that Gln$_{92}$ acts as an essential residue for high reactivity of GPx7 with H$_2$O$_2$ and hence effective oxidation of PDI by the enzyme.

**GPx7 Cys$_{86}$ plays a critical role in the regulated oxidative activity of GPx7**

Three-dimensional structures of reduced forms of GPx7 and GPx8 have high similarity, in which the locations and orientations of C$_P$ and C$_R$ in GPx7 are almost identical to those in GPx8 (29). The S$_y$ atoms of C$_P$ and C$_R$ are separated by $\sim$11 Å in both the enzymes, suggesting that a large conformational change is required for formation of an intramolecular disulfide bond between these two cysteines (30). Given the situations, two alternative mechanisms have been proposed for GPx7/GPx8-mediated PDI oxidation: a one-cysteine mechanism in which the CP acts as the sole redox center that reacts with both H$_2$O$_2$ and PDI and a two-cysteine mechanism in which an intramolecular disulfide bond between CP and CR is formed preceding the oxidation of PDI (30, 33).

To investigate which of the mechanisms is primarily exerted by GPx7 and GPx8, we constructed single-Cys mutants of the enzymes in which C$_R$ was mutated to alanine: C86A for GPx7 and C108A for GPx8. Of interest, the results demonstrated that GPx7 C86A oxidized PDI more efficiently than GPx7 WT (Figs. 2A, left middle panel, and 4A, left panel), suggesting that a one-cysteine mechanism is more competent in PDI oxidation than a two-cysteine mechanism and that GPx7 WT exerts primarily a
two-cysteine mechanism or both one- and two-cysteine. By contrast, GPx8 C108A oxidized PDI even less efficiently than GPx8 WT (Figs. 2A, middle right panel, and 4A, right panel), indicating the necessity of CR for the GPx8-mediated PDI oxidation. Thus, GPx8 is likely to utilize a two-cysteine mechanism exclusively.

For GPx7 to exert a one-cysteine mechanism with high efficiency, its CP must be very reactive with H2O2. To verify that it is indeed the case, we investigated the susceptibility of GPx7 C86A to H2O2-mediated hyperoxidation. As expected, GPx7 C86A was rapidly hyperoxidized with 50 μM H2O2, whereas its counterpart, GPx8 C108A, was much less sensitive to H2O2 (Fig. 4B). To further compare the oxidative activities of GPx7 WT and C86A, we investigated oxidative folding of RNase A catalyzed by these two using gel-shift assay (Fig. 4C). The results demonstrated that GPx7 C86A oxidized RNase A much more rapidly than GPx7 WT. Accordingly, GPx7 C86A restored RNase A activity more quickly than GPx7 WT (Fig. 4D). Thus, GPx7 can be a more competent H2O2-dependent oxidase by exerting a one-cysteine mechanism exclusively on the highly reactive peroxidatic cysteine.

To explore whether GPx7 WT primarily employs a one- or two-cysteine mechanism, we examined the PDI concentration dependence of the rate of PDI oxidation by GPx7 WT and C86A.

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**Figure 2. GPx7/GPx8-catalyzed oxidation of PDI in the presence of hydrogen peroxide.**

A. time course of the redox-state changes of PDI during noncatalyzed, GPx7-catalyzed, and GPx8-catalyzed PDI oxidation. All experiments were initiated by mixing 10, 50, and 200 μM H2O2 into the mixture of reduced PDI (10 μM) and GPx7 or the luminal domain of GPx8 (1 μM) in buffer containing 50 mM Tris/HCl (pH 7.4) and 300 mM NaCl. At the indicated time points, the reaction mixture was alkylated with mal-PEG 2k. The redox states of PDI were separated by nonreducing SDS-PAGE and stained with CBB. Red. PDI, Part. Oxi., and Oxi. PDI denote fully reduced, partially oxidized, and fully oxidized species of PDI, respectively. B. time course of the redox-state change of GPx7 WT (1 μM) during the catalysis of oxidation of PDI (10 μM) in the presence of 50 or 200 μM H2O2. A representative gel image of three experiments at each condition is displayed in both A and B.
mechanism gets more predominant over a two-cysteine mechanism because, under such conditions, reduced PDI should more likely win the race of nucleophilic attack to a sulfenylated CP against the CR residue of the same GPx7 molecule (Fig. 5A). Given that, the oxidative activity of GPx7 WT will approach that of GPx7 C86A as the concentration of PDI increases. However, higher PDI concentrations did not render the initial NADPH consumption rates of GPx7 WT and C86A closer to each other (Fig. 5, B and C). It is thus conceivable that, regardless of the PDI concentration, GPx7 WT primarily employs a two-cysteine mechanism, resulting in the slower oxidation of PDI and hence the slower oxidative folding of a downstream substrate than GPx7 C86A (Fig. 4).

**Figure 3. Effects of a mutation at the catalytic tetrad on the reactivity of GPx7/8 with hydrogen peroxide and PDI.**

A, amino acid sequence alignment in the CP- and Cx-neighboring regions of human GPx7 and GPx8. B, catalytic tetrad of GPx7 and GPx8 involving a peroxidatic cysteine and its neighboring Trp and Gln/Ser residues. In GPx7, the Oe1 atom of the Gln92 side chain is distant from the Sg atom of Cys57 by 3.1 Å, whereas the Oe1 atom of Ser114 in GPx8 side chain is separated from the Sg atom of Cys79 by 4.8 Å. C, time course of the redox-state changes of GPx7 and GPx8 swap mutants (GPx7 Q92S and GPx8 S114Q) during a reaction between 1 μM GPx7/8 and 10 or 50 μM H2O2. At the indicated time points, the reaction mixture was alkylated with mal-PEG 2k. D, time course of the redox-state change of PDI during a reaction between 1 μM GPx7 swap mutant (GPx7 Q92S) or GPx8 swap mutant (GPx8 S114Q) and 10 μM PDI in the presence of 10 or 50 μM H2O2. A representative gel image of three experiments at each condition is displayed in C and D.

**Biochemical characterizations of GPx7 and GPx8**

Previous studies by us and others suggest that PDI oxidases have high selectivity against the PDI family members in the disulfide bond formation network (10, 12, 21, 29, 44, 45). The
present study indicated that whereas the PDI oxidation activity of GPx8 is minimal, GPx7 has significant PDI oxidation activity. To investigate GPx7 preference for the PDI family members, five representative members of the PDI family (PDI, ERp46, P5, ERp57, and ERp72), either independently or in a mixture of five, were subjected to oxidation by GPx7 in the presence of H2O2. At the indicated time points, the reaction mixture was alkylated with mal-PEG 2k. Red. and Hyper Oxi. denote fully reduced and hyperoxidized species of the GPx7/8 mutants, respectively. C, time course of the redox state changes of reduced/denatured RNase A (20 μM) during oxidation by 10 μM PDI and 1 μM GPx7 WT or C86A in the presence of H2O2. At the indicated time points, the reaction mixture was alkylated with AMS. Red. RNase A, Part. Oxi. RNase A, and Oxi. RNase A denote fully reduced, partially oxidized, and fully oxidized species of RNase A, respectively. A representative gel image of three experiments at each condition is displayed in A, B, and C. D, recovery of RNase A activity during oxidative folding catalyzed by 10 μM PDI and 1 μM GPx7 WT or C86A in the presence of H2O2 (n = 3, means ± S.D.).

**Figure 4. Effects of a Cᵦ-residue mutation on the oxidative activities of GPx7/8.**

A, time course of the redox-state change of PDI during a reaction between 1 μM GPx7 C86A or GPx8 C108A and 10 μM reduced PDI in the presence of 50 μM H2O2. At the indicated time points, the reaction mixture was alkylated with mal-PEG 2k. Red. PDI and Oxi. PDI denote fully reduced and hyperoxidized species of the GPx7/8 mutants, respectively. B, time course of the redox-state change of GPx7 C86A and GPx8 C108A after mixture with 50 μM H2O2. At the indicated time points, the reaction mixture was alkylated with mal-PEG 2k. Red. and Hyper Oxi. denote fully reduced and hyperoxidized species of the GPx7/8 mutants, respectively. C, time course of the redox state changes of reduced/denatured RNase A (20 μM) during oxidation by 10 μM PDI and 1 μM GPx7 WT or C86A in the presence of 50 μM H2O2. At the indicated time points, the reaction mixture was alkylated with AMS. Red. RNase A, Part. Oxi. RNase A, and Oxi. RNase A denote fully reduced, partially oxidized, and fully oxidized species of RNase A, respectively. A representative gel image of three experiments at each condition is displayed in A, B, and C. D, recovery of RNase A activity during oxidative folding catalyzed by 10 μM PDI and 1 μM GPx7 WT or C86A in the presence of 50 μM H2O2 (n = 3, means ± S.D.).

**Biochemical characterizations of GPx7 and GPx8**

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H2O2-induced complex formation between PDIs and GPx7/8 in cells

To gain insights into physiological redox partners of GPx7 and GPx8, we next transfected HeLa cells with plasmids
expressing FLAG-tagged Prx4, GPx7, or full-length GPx8; treated the cells with or without 0.5 mM H2O2 for 10 min; and alkylated the free cysteines with N-maleimide (NEM) as described under “Experimental procedures,” followed by immunoprecipitation and immunoblotting with an antibody against each PDI family member (Fig. 7). We thus reproduced our previous observation that Prx4 most preferentially bound ERp46 and P5 in an H2O2-independent manner in cells (10). Notably, GPx7 bound PDI only slightly and did not bind the other PDI family members without H2O2 (Fig. 7). However, H2O2 treatment significantly increased amounts of PDI and P5 bound to GPx7. A similar observation was made with GPx8 despite even smaller amounts of PDI and P5 co-immunoprecipitated with GPx8 (Fig. 7). Thus, GPx7 displayed PDI preference different from Prx4,

Figure 5. Effects of PDI concentration on the oxidative activities of GPx7 WT and C86A. A, alternative (one- or two-cysteine) mechanism of GPx7 in the catalysis of PDI oxidation. A peroxidatic cysteine (CP) of GPx7 is readily sulfenylated upon reaction with H2O2, followed by the race between reduced PDI and a resolving cysteine (CR) of the same GPx7 molecule against the sulfenylated CP. B, NADPH consumption coupled to PDI oxidation catalyzed by GPx7 WT or GPx7 C86A was monitored by measuring absorbance change at 340 nm. All experiments were initiated at 30 °C by mixing 50 μM H2O2 into the mixture of reduced PDI (5–50 μM), GSH (1 mM), GR (1 unit), NADPH (200 μM), and GPx7 WT or GPx7 C86A (1 μM) in buffer containing 50 mM Tris/HCl (pH 7.4) and 300 mM NaCl. Representative data of three experiments at each condition are displayed. C, plots of the initial NADPH consumption rate (mM/min) during the catalysis of GPx7 WT or GPx7 C86A as a function of PDI concentrations. Note that the rates were calculated by subtracting the rate of noncatalyzed reaction (No GPx7) from those of GPx7-catalyzed reactions (GPx7 WT or C86A) (n = 3, means ± S.D.).
although both the peroxidases bound their preferential PDIs more tightly in the presence of H₂O₂. Notably, the intracellular redox partners of GPx7 did not coincide with its preferential substrates identified by \textit{in vitro} experiments (Fig. 6), suggesting the presence of mediator proteins that link GPx7 to its physiological redox partners (see also “Discussion”).

\textbf{Discussion}

Previously, GPx7 and GPx8 were demonstrated to be capable of utilizing Ero1α-derived H₂O₂ in cells (29). Although GPx8 was reported to interact directly with Ero1α and scavenge the neighboring H₂O₂ molecule (39), the present work revealed that GPx7 had much higher reactivity with H₂O₂ than GPx8, leading to the more efficient PDI oxidation. Although the affinity for H₂O₂ of GPx7 was not precisely determined in this study, 10 μM H₂O₂ was sufficient to oxidize GPx7 rapidly (Fig. 1). It is thus conceivable that GPx7 could compete with Prx4, another ER-resident peroxidase with a micromolar range of affinity for H₂O₂, for reacting with H₂O₂ if these two enzymes co-localize in the same compartment of the ER. However, considering that unlike GPx7, Prx4 does not scavenge Ero1α-generated H₂O₂ efficiently in cells (39), these two enzymes seem likely to have distinct physiological roles in the ER.

Reactivity of a given cysteine residue depends on the propensity of its thiolate form, which is quantitatively expressed as pKₐ (46). However, pKₐ is not the sole factor that determines the reactivity of a peroxidatically active cysteine with H₂O₂ (43, 47). The active sites of GPx7 and GPx8 consist of a catalytic tetrad including Cys, Trp, Asn, and Gln/Ser residues. We here demonstrated that the Gln92 residue in the catalytic tetrad of GPx7 is essential for the enhanced reactivity of GPx7 CP with H₂O₂, probably because Gln92 serves to stabilize the sulfenylated CP species via hydrogen bonding (Figs. 3 and 4B). Gln92 is highly conserved among all GPx family members but GPx8, in which Gln is substituted by Ser114. In this regard, Ser114 can be interpreted to have an inhibitory role in the H₂O₂-scavenging activity of GPx8 (39), possibly providing GPx8 with other physiological functions in cells.

The present study also demonstrates that GPx7 WT preferentially employs a two-cysteine mechanism in oxidizing PDI, whereas the GPx7 C86A mutant oxidizes PDI at a much higher
Figure 7. Complex formations of Prx4, GPx7 and GPx8 with PDI family members in cells. A, HeLa cells were transfected with pcDNA3.1 (empty vector) (lanes 1, 2, 9, and 10), pCMV-Gg PRX4 (encoding PRX4-FLAG) (10) (lanes 3, 4, 11, and 12), pES104 (encoding GPx7-FLAG) (lanes 5, 6, 13, and 14), or pES105 (encoding full-length GPx8-FLAG) (lanes 7, 8, 15, and 16), grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h, treated with 0.5 mM H$_2$O$_2$ (lanes 9–16) for 10 min or not (lanes 1–8), washed twice with PBS, treated with 10% TCA, and subjected to alkylation with NEM (51). The NEM-treated cell lysates were subjected to immunoprecipitation with anti-FLAG M2 magnetic beads (Sigma) to purify complexes containing FLAG-tagged proteins. The immunoprecipitates were separated by reducing SDS-PAGE, and PDI family members and FLAG-tagged constructs contained in the immunoprecipitates were detected with antibodies to the indicated PDI family members or horseradish peroxidase-conjugated antibody to FLAG. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 contained 1 μg of NEM-treated cell lysate (Input). Lanes 2, 4, 6, 8, 10, 12, 14, and 16 contained immunoprecipitates (IP) obtained from 50 μg of NEM-treated cell lysate. The positions of the FLAG-tagged constructs were indicated on the right in the bottom panel. B, the band intensities of PDI family members in the input and immunoprecipitate lanes on A were quantified using ImageJ 1.50i. PDI family members immunoprecipitated with each FLAG-tagged peroxidase are shown as a percentage of total PDIs in the cell lysate. The experiments were performed once.
Biochemical characterizations of GPx7 and GPx8

Experimental procedures

Plasmids

Plasmids for overexpression of GPx7 and the luminal domain of GPx8 in Escherichia coli (pKEHS780 and pVD54, respectively) (29) were kind gifts from Dr. Lloyd Ruddle (University of Oulu). GPx7 and GPx8 Cys–Ala mutants were constructed by using the QuickChange site-directed mutagenesis kit (Agilent Technologies).

Primers and templates used to construct following plasmids are listed in Table 1. Plasmid pES101 (encoding GPx7) was constructed by assembling three DNA fragments using the Gibson assembly kit (New England Biolabs). The DNA fragments used were a fragment amplified from pcDNA3.1+ (Invitrogen) with primers NeoRH1 and gpx7E1, a fragment amplified from pKEHS780 with primers gpxE72 and gpxE4, and a fragment amplified from pcDNA3.1+ with primers NeoRH2 and gpxE3. Plasmid pES102 that expresses the luminal domain of GPx8 in the ER and plasmid pES103 (encoding GPx8) were constructed by assembling fragments amplified using a template and a primer set indicated on Table 2. Plasmids pES104 (encoding GPx7-FLAG on pcDNA3.1+) and pES105 (encoding GPx8-FLAG on pcDNA3.1+) were constructed by inserting a triple FLAG sequence in front of the KDEL sequence of these proteins. They were constructed also by assembling fragments amplified using a template and a primer set indicated on Table 2.

Protein overexpression and purification

Plasmids for overexpression of GPx7, the luminal domain of GPx8, PDIS, and their mutants were transformed into E. coli BL21(DE3). Cells harboring the plasmids were grown at 30 °C and induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside at an A600 of 0.5. The cells were cultured for an additional 4 h at 30 °C and then harvested. To purify GPx7, GPx8, and their mutants, cells in buffer A (50 mM Tris/HCl, pH 8.1, 0.3 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) were disrupted using a microfluidizer (Niro Soavi PA2K). After centrifugation of the cell lysate to remove cellular components at 12,000 rpm for 20 min, the supernatant was loaded onto an open nickel–nitrilotriacetic acid–Sepharose column (Qiagen). The column was washed with buffer A containing 20 mM imidazole, and the proteins were eluted with buffer A containing 200 mM imidazole. The eluted sample was concentrated to 500 µl by filtration using Amicon Ultra filter units (molecular weight cutoff, 10,000; Millipore), applied to a Superdex 75 size-exclusion column (GE Healthcare) pre-equilibrated with 50 µM Tris/HCl (pH 8.1), 0.3 mM NaCl, and 1 mM EDTA and finally eluted with the same buffer.

To purify PDIS, the supernatant of the cell lysate was applied to the open nickel–nitrilotriacetic acid–Sepharose column. Fractions eluted with 200 mM imidazole were further purified...
Biochemical characterizations of GPx7 and GPx8

Table 1

| Plasmids   | Fragment 1 | Fragment 2 | Fragment 3 |
|------------|------------|------------|------------|
| pES101     | pcDNA3.1.1  | NeoRH1 + gpxE7E1 | pcDNA3.1.1  |
| pES102     | pcDNA3.1.1  | NeoRH1 + gpxE7E1 | pcDNA3.1.1  |
| pES103     | pES101     | NeoRH1 + gpxE8E5 | NeoRH2 + gpxE7E3 |
| pES104     | pES101     | NeoRH1 + gpxE7E6 | NeoRH2 + gpxE7E3 |
| pES105     | pES103     | gpxE8E10    | NeoRH2 + gpxE7E3 |

by MonoQ anion exchange column (GE Healthcare) and Superdex 200 size-exclusion column (GE Healthcare).

Antibodies, reagent, and immunological techniques

Rabbit antibodies to ERp57 (GTX100297), and to ERp72 (GTX115263) were purchased from GeneTex. For the detection of proteins fused with a triple FLAG tag, mouse anti-FLAG M2 antibody conjugated with horseradish peroxidase (Sigma Aldrich) was used. Rabbit antibody to PDI has been published (50). Antibodies to ERp46 and P5 were used as described previously (10). Immunoblotting was performed using standard methods. The protein bands were visualized using an appropriate secondary antibody, Clarity Western ECL substrate (Bio-Rad) and ChemiDoc touch imaging system (Bio-Rad). The images were processed with Image laboratory software (Bio-Rad).

Analysis of the redox states of GPx7, GPx8, PDI, and their mutants

Purified GPx7, the luminal domain of GPx8, PDIs, and their mutants were separately reduced with 10 mM DTT for 10 min at 30 °C followed by DTT removal through a PD-10 column (GE Healthcare) pre-equilibrated with 50 mM Tris/HCl (pH 7.4) buffer containing 300 mM NaCl. RNase A from bovine pancreas (Sigma–Aldrich) was reduced and denatured with 100 mM DTT and 6 K guanidinium hydrochloride. The sample was passed through a PD-10 column (GE Healthcare) pre-equilibrated with 10 mM HCl aq. to remove the reducing and denaturing reagents. Reduced/denatured RNase A was diluted to 20 μM (~50-fold dilution) and incubated with 1 μM GPx7 or its mutant and 10 μM PDI in a buffer (50 mM Tris/HCl, pH 7.4, and 300 mM NaCl) at 30 °C, and the reactions were initiated by adding 50 μM of H₂O₂. At the indicated time points, the samples were quenched using 1 mM mal-PEG 2000 (NOF Corporation). The reaction mixture was boiled for 3 min after addition of an equal volume of 2× Laemmli buffer. All of the samples were run through nonreducing SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) (Nacalai Tesque). The gel images were captured by using ChemiDoc touch imaging system (Bio-Rad).

Oxidative folding assay of RNase A

RNase A from bovine pancreas (Sigma–Aldrich) was reduced and denatured with 100 mM DTT and 6 K guanidinium hydrochloride. The sample was passed through a PD-10 column (GE Healthcare) pre-equilibrated with 10 mM HCl aq. to remove the reducing and denaturing reagents. Reduced/denatured RNase A was diluted to 20 μM (~50-fold dilution) and incubated with 1 μM GPx7 or its mutant and 10 μM PDI in a buffer (50 mM Tris/HCl, pH 7.4, and 300 mM NaCl) at 30 °C, and the reactions were initiated by adding 50 μM of H₂O₂. At the indicated time points, the samples were quenched using 1 mM 4-acetamido-4′-maleimidostilbene-2,2′-disulfonic acid (AMS; Molecular Probes, Inc.). All samples were separated by nonreducing SDS-PAGE followed by staining with CBB. The gel images were captured by the ChemiDoc touch imaging system (Bio-Rad). The recovery of RNase A activity was measured by monitoring the linear decrease in absorbance at 295 nm on a U-3900 spectrophotometer (Hitachi) at 30 °C after addition of 1.25 mM cytidine-2′,3′- cyclic monophosphate monosodium salt (Sigma–Aldrich) to reaction mixtures at the indicated time points.

NADPH consumption assay

NADPH consumption coupled with GPx7-catalyzed PDI oxidation was monitored by measuring absorbance change at 340
nm using a SH-9000 microplate reader (Corona Electric Co.). The solution was first prepared by mixing 1 μM GPx7 WT or GPx7 C86A, 5–50 μM reduced PDI, 1 mM GSH, 1 unit GSH reductase (GR), and 200 μM NADPH in a buffer (50 mM Tris/HCl, pH 7.4, and 300 mM NaCl), and the reactions were initiated at 30°C by adding 50 μM of H₂O₂ to the solution. The initial NADPH consumption rates were calculated by considering the decrease of absorbance at 340 nm during the initial reaction time of 30 s with a molar extinction coefficient value of 6200 M⁻¹ cm⁻¹ for NADPH.

**Preferential PDI oxidation by GPx7 in vitro**

5 μM each of purified PDI family members (PDI, P5, ERp46, ERp57, and ERp72) was mixed with 0.5 μM GPx7. The reactions were initiated by adding 50 μM of H₂O₂ to the solutions. At the indicated time points, the samples were subjected to 2 mM mal-PEG 2000 (NOF Corporation) followed by immunoblotting with the corresponding anti-PDI antibodies.

**Cell culture, transfection, and sample preparation**

The cells were transfected with plasmids using Effectene (Qiagen). For the transfection, HeLa cells were plated in 10-cm dishes at 8 × 10⁵ cells/well, cultured for 24 h, and transfected with an appropriate plasmid, following the manufacturer’s instruction. The amount of plasmid DNA used for the transfection was 2 μg for pcDNA3.1⁺ and pCMV-Gg PRX4 (encoding PRX4-FLAG) (10), 6 μg for pES104 (encoding GPx7-FLAG), and 6 μg for pES105 (encoding full-length GPx8-FLAG). The larger amount of plasmid DNA was used for transfection with the latter two plasmids because the levels of protein production from these plasmids were smaller than that from the former one when the same amount of plasmid DNA was used. At 24 h after the transfection, the cells were treated with or without 0.5 mM H₂O₂ for 10 min, washed twice with PBS, treated with 10% TCA, and subjected to alkylation with NEM essentially as described (51).

**Immunoprecipitation**

500 μg of NEM-treated cell lysate prepared as described above was diluted with ice-cold KI buffer (2% (w/v) Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and centrifuged at 15,000 × g for 10 min at 4°C to obtain NEM-treated cleared cell lysate. To purify FLAG-tagged proteins (PRX4-FLAG, GPx7-FLAG, or GPx8-FLAG) from the cleared cell lysate, 50 μl of prewashed anti-FLAG M2 magnetic beads (Sigma) were incubated with the NEM-treated cleared cell lysate at 4°C for 3 h. The immune complexes were collected by magnetization and washed four times with 800 μl of ice-cold high-salt buffer (1% (w/v) Triton X-100, 50 mM Tris-HCl, pH 8.0, 1 mM NaCl, 1 mM EDTA) and once with 800 μl of 10 mM Tris-HCl (pH 8.0). The immunoisolates were then released by incubating the sample at 37°C for 1 h with 65 μl of 2× Laemmli sample buffer supplemented with 10 mM NEM, 2 μg/ml pepstatin A, 1 mM benzamidine, and 1 mM phenylmethylsulfonfyl fluoride.

**Biochemical characterizations of GPx7 and GPx8**

**Data availability**

All data described are contained within the article.

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**Abbreviations**—The abbreviations used are: ER, endoplasmic reticulum; PDI, protein-disulfide isomerase; GPx7/8, glutathione peroxidase-7/8; Prx4, peroxiredoxin-4; CP, peroxidatic cysteine residue; CR, resolving cysteine residue; mal-PEG 2k, maleimidyl PEG-2000; NEM, N-maleimide; CBB, Coomassie Brilliant Blue; AMS, 4-acetamidine-4’-maleimidylstilbene-2,2’-disulfonic acid; GR, glutathione reductase.

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