Thioredoxin 1 is inactivated due to oxidation induced by peroxiredoxin under oxidative stress and reactivated by glutaredoxin system*

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*Running title: Trx1 is inactivated by Prx1 and reactivated by Grx system

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Background: The Cys62/Cys69 dithiol of Trx1 is predicted to have a profound effect on cell signaling.

Results: The Cys62/Cys69 dithiol of Trx1 was oxidized by Prx1 and this disulfide was reduced by GSH/Grx system.

Conclusion: Trx1 is involved in redox regulation via reversible oxidation of the Cys62/Cys69 dithiol of Trx1.

Significance: We demonstrated the critical role of Trx1 oxidation in cell signaling.

Summary

The mammalian cytosolic thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is the major protein-disulfide reductase of the cell and has numerous functions. Besides the active site thiols, human Trx1 contains three non-active site cysteine residues in positions 62, 69 and 73. A two-disulfide form of Trx1, containing an active site disulfide between Cys32 and Cys35 and a non-active site disulfide between Cys62 and Cys69, is inactive either as a disulfide reductase or as a substrate for TrxR. This could possibly provide a structural switch affecting Trx1 function during oxidative stress and redox signaling. We found that the two-disulfide form Trx1 was generated in A549 cells under oxidative stress. In vitro data showed that the two-disulfide form Trx1 was generated from oxidation of Trx1 catalyzed by peroxiredoxin 1 in the presence of H₂O₂. The redox western blot data indicated that glutaredoxin system protected Trx1 in HeLa cells from oxidation caused by ebselen, a superfast oxidant for Trx1. Our results also showed that physiological concentrations of glutathione, NADPH and glutathione reductase reduced the non-active site disulfide in vitro. This reaction was stimulated by glutaredoxin 1 via the so-called mono-thiol mechanism. In conclusion, reversible oxidation of the non-active site disulfide of Trx1 is suggested to play an important role in redox regulation and cell signaling via temporal inhibition of its protein-disulfide reductase activity for the transmission of oxidative signals under oxidative stress.

Thioredoxins are a family of small proteins catalyzing thiol-disulfide oxidoreductions by
using redox-active cysteine residues in their active site (WCGPC), which is conserved among species from cyanobacteria to humans (1-4). Thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH compose the Trx system, which operates by transferring electrons from NADPH via TrxR to the active site of Trx. The active site cysteines of Trx are readily accessible on the surface of the protein and become oxidized to a disulfide upon reduction of a target protein. This disulfide is cycled back to dithiol by TrxR (4,5). Trx is the major disulfide reductase with a large number of functions in cells. These involve DNA synthesis via ribonucleotide reductase, reduction of methionine sulfoxide reductase and particularly defense against oxidative stress by providing the electrons to peroxiredoxins (Prx). In addition, Trx in its reduced form plays important roles in redox regulation of many transcription factors such as nuclear factor-κB (NF-κB), redox factor-1 (Ref-1), apoptosis signaling kinase (ASK1), and activator protein-1 (AP-1) (4,6,7). For example, reduced Trx1 can bind and inactivate ASK1, a mitogen-activated protein kinase–kinase–kinase which can induce apoptosis (8). In vivo Trx activity may be regulated by binding to thioredoxin-interacting protein (Txnip) (9-11).

Mammalian cells contain two distinct Trxs. Trx1 is localized in cell cytosol/nucleus, whereas Trx2 is a mitochondrial protein (4). In addition to the active site Cys32 and Cys35, the mammalian Trx1 has three non-active site cysteine residues (Cys62, Cys69 and Cys73), which have been suggested to be important for regulating the activity and function of Trx1 (12). Cys62 and Cys69 are within the α3 helix, and Cys73 is on a hydrophobic patch of surface of the protein. Cys73 is present as an intermolecular disulfide bond (Trx1 homodimer) in x-ray crystal studies (13,14). The mass spectrometry data reveals a second intramolecular disulfide besides the active site disulfide, formed between Cys62 and Cys69 under oxidizing conditions (14). The formation of the second disulfide between Cys62 and Cys69 impairs Trx1 activity and is predicted to have a profound effect by disrupting interactions between Trx and its target protein (6,14,15). In vitro, a two-disulfide form of Trx1 (Trx1-S4) is inactive either as a disulfide reductase, or as a substrate for TrxR, and is no longer an antiapoptotic factor via ASK1, supporting the interpretation that oxidation of the non-active site dithiol could possibly provide a structural switch affecting Trx1 function during oxidative stress and redox signaling (14,16). However, how this non-active site disulfide is generated and fulfills its biologic functions in redox signaling are unknown so far.

H₂O₂ was viewed as the inevitable but unwanted by-product of oxidative metabolism in cells. Prxs exert their protective antioxidant role in cells through their peroxidase activity, whereby H₂O₂ is reduced and scavenged. However, nowadays H₂O₂ has been revealed to possess important functions in cell signaling as a second messenger (11,17). In cytosol of mammalian cell, the Prxs appear to be involved in the redox regulation of cellular signaling and differentiation by regulating the levels of H₂O₂ (18-20). However, the detailed mechanism on how H₂O₂ exerts its activity in redox regulation of cell signaling is still unclear.

In this study, we found that the oxidation of Trx1 was catalyzed by Prx1 in the presence of H₂O₂, suggesting that the non-active site cysteines were able to transfer electrons to Prx1 and therefore possibly had a reduction activity. The Grx system could reduce the non-active site disulfide of Trx1 which was not a substrate of TrxR. Therefore, Trx1 might be involved in redox regulation of cellular signaling via the temporal inactivation of its redox activity due to the reversible oxidation of the non-active site cysteines under oxidative stress.

**EXPERIMENTAL PROCEDURES**
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**Chemicals and proteins** – Buthionine sulfoximine (BSO), dithiothreitol (DTT), GSH, iodoacetic acid (IAA), iodoacetamide (IAM), insulin and glutathione reductase were purchased from Sigma-Aldrich. Ebselen was a product of Daiichi Pharmaceutical Co. (Tokyo, Japan). Wild type human Trx1, human glutaredoxin 1, *E. coli* glutaredoxin 1 C14S mutant protein and human Trx1 antibody were products of IMCO Ltd. (Stockholm, Sweden). Recombinant TrxR1 was a gift from Dr. Elias Arnér, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, and was purified as described before (21).

**Cell Culture** - Human alveolar adenocarcinoma epithelial A549 cells and cervical carcinoma HeLa cells were cultured in Dulbecco’s Modified Eagle medium (Biochrom, Germany) supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in an incubator with 5% CO₂.

**Detection of Trx1 Redox State in Cells** – The redox state of Trx1 was detected using modified redox western blot developed from (22-24). To prepare mobility standards, cell lysates were denatured and unfolded with urea and fully reduced with DTT. Then varying molar ratios of IAA to IAM were incubated with the reduced proteins containing n cysteines, producing n+1 protein isoforms with introduced number of acidic carboxymethyl thiol adducts (-SA⁺) and neutral amidomethyl thiol adducts (-SM). During Urea-PAGE, the ionized -SA⁺ adducts resulted in faster protein migration toward the anode. Therefore, the n+1 isoforms were separated and used as a mobility standard for representing the number of -SA⁺. To determine the redox state of Trx1 in vivo, cells were washed twice with PBS and lysed in 300 µl of urea lysis buffer (50 mM Tris-HCl, pH 8.3/1 mM EDTA/8 M urea) containing 10 mM IAM. Then free thiols were alkylated by IAM at 37°C for 20 min. After removing the cell debris by centrifugation, the cell lysates were precipitated by ice-cold acetone-HCl. The precipitate was washed with ice-cold acetone-HCl two more times and resuspended in 100 µl of urea lysis buffer containing 3.5 mM DTT. After incubation at 37°C for 30 min, 5 µl of 600 mM IAA was added to each sample, and incubated for 30 min at 37°C. Then protein concentration was determined by Lowry protein assay and equal amounts of protein were separated by urea-PAGE gel and blotted to nitrocellulose membrane (Bio-Rad, USA). Membranes were probed with the appropriate primary antibody, biotinylated secondary antibody (Dako, Denmark) and Streptavidin-HRP anti-biotin tertiary antibody (Thermo-Pierce, USA), and then visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, USA).

**Construction of plasmid for human Trx1 mutant (Trx1SGPS)** - Primers to construct the C32S/C35S (SGPS) mutant of pET-16b/Trx were from Thermo Fisher Scientific. The forward primer was: 5’-GC GGT CTC GGG CCT TCC AAA ATG ATC AAG CCT TTC-3’ and the reverse primer was: 5’- GC GGT CTC AGG CCC AGA CCA CGT GGC TGA GAA GTC-3’. The site-directed mutagenesis plasmid was constructed using inverse PCR method (25). Existence of the mutation was verified by sequencing.

**Purification of recombinant human Prx1 and Trx1SGPS** - *E. coli* strain BL21 was transformed with the plasmid pNIC-Bsal encoding *Homo sapiens* Prx1 (NM_181696) or the plasmid pET16b encoding Trx1SGPS. Proteins were expressed by auto-induction method (26) at 20°C in auto-induction TB medium. Cultures were collected and lysed in 50 mM Tris-HCl buffer, and then the clear lysates were subjected to a nickel affinity chromatography for protein purification.

**Redox state shift of human Trx1-S₂ and Trx1SGPS in the presence of Prx1 and/or H₂O₂** -
Trx1-S2, which contains an active site disulfide and three non-active site thiols, was prepared as follows. 0.6 mM human Trx1 in TE buffer was reduced by 3.5 mM DTT, and then the sample was desalted on a Sephadex G25 gel filtration column to remove excess DTT. 90 μM reduced Trx1 was incubated with 45 μM insulin at room temperature for 30 min, and then the sample was spun at 16000 X g for 5 min to remove precipitated insulin. The redox state of Trx1-S2 was verified by redox urea-PAGE gel. 10 μM of Trx1-S2 or reduced Trx1SGPS was incubated with various concentrations of Prx1/H2O2 for 5 min at 37°C. Then the reactions were stopped by adding 10 mM IAM in the reaction solutions, which also fixed the redox state of Trx1 at the same time. These samples were subjected to a redox urea-PAGE gel and visualized by coomassie blue staining.

Activity assay for reduction of oxidized Trx1 - Human Trx1 and Trx1SGPS protein were oxidized with 5 mM H2O2 for 10 min at room temperature, and then desalted using a Sephadex G25 gel filtration column. In a cuvette, the assay system was comprised of 0.25 mM NADPH, 45 nM glutathione reductase, and indicated amounts of GSH and Trx1/Trx1SGPS, with or without Grx1 in TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH7.5). Consumption of NADPH was monitored by monitoring the absorbance change at 340 nm.

RESULTS
Cellular redox state response of Trx1 to oxidative stress
So far the precise mechanism of the transmission of oxidative signals under oxidative stress was not well known. The role of Trx1 in the redox signaling under oxidative stress was explored in our study. We treated A549 cells with high concentration of H2O2 as oxidative stress source and then redox state of Trx1 in cells was detected using modified redox western blot method. In the classic redox western blot (22-24), cells were lysed by 8 M urea containing IAA for alkylation the free thiols of Trx1. However, the rate for the alkylation of Trx1 with IAM was found to be 20-fold faster than that with IAA (27). Therefore, we used IAM instead of IAA for alkylation the free thiols of Trx1 in the modified method, which would be beneficial to decrease the risk of oxidation of Trx1 during the cell lysis process. It should be noted that the nitrosylated or glutathionylated cysteines behave the same as the cysteines forming disulfide bonds in the redox western blot assay. Therefore, in Table 1, we summarized the most likely forms of Trx1 for each band shown in this assay.

As shown in Fig. 1, physiological state of Trx1 in the untreated cells was fully reduced, in line with the previous reports (24). Under oxidative stress, redox state of Trx1 shifted from fully reduced state to oxidized state. It should be noted that the two disulfides form of Trx1 with or without a free thiol was observed under oxidative stress. As shown in Table 1, band 0 represents the form containing two disulfides (Cys32/Cys35 and Cys62/Cys69) and a modified cysteine (Cys73). The modification of Cys73 residue might be glutathionylation, nitrosylation or the disulfide between Cys73 of two Trx1 molecules (7). But in any case, the non-active site disulfide was resistant to regeneration by TrxR1, and therefore the two disulfides form of Trx1 was inactive, which would allow time for redox dependent signaling processes to occur.

In vitro oxidation of the non-active site thiols of Trx1 in the presence of Prx1 and/or H2O2
Because H2O2 is reduced and scavenged by Prx1 using Trx1 as the electron donor, it is interesting to understand the effect of H2O2 on formation of the non-active site disulfide of Trx1 in the presence/absence of Prx1. First, we prepared the oxidized wild type Trx1 containing an active site disulfide and three non-active site thiols (Trx1-S2) as described in methods section, which was verified by redox gel as shown in the upper panel of Fig. 2, lane 1. Then physiological
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concentration of Trx1-S₂ was incubated with H₂O₂ in the presence/absence of Prx1. As shown in the upper panel of Fig. 2, H₂O₂ was not an effective oxidant for the formation of the non-active site disulfide of Trx1 in the absence of Prx1 (lane 2 and 4). However, in the presence of Prx1, the formation of Trx1-S₄ (band 1 and band 0 in lane 3 and 5) was strongly stimulated, indicating that H₂O₂ indirectly induced formation of Trx1-S₄ via Prx1. The similar result was observed in the lower panel of Fig. 2 using pre-reduced Trx1SGPS, which only contained three of non-active site thiols.

**GSH system protected cellular Trx1 from oxidation**

GSH system is another important antioxidant system besides Trx system in cells. In our study, HeLa cells were pre-treated with BSO, which was an inhibitor of γ-glutamylcysteine synthetase, to deplete the cellular GSH. Then the cells with or without BSO pre-treatment were incubated with ebselen, which is a substrate for both TrxR1 and Trx1, and a superfast oxidant for Trx1 (28). As shown in Fig. 3, most of Trx1 in the cells without BSO pre-treatment remained in the fully reduced state after incubation with ebselen (lane 2-4). However, after incubation with ebselen, redox state of Trx1 in the cells with BSO pre-treatment shifted from fully reduced state to oxidized state (lane 5-7), indicating that GSH system protected Trx1 from oxidation in vivo.

**GSH system was an electron donor for reduction of the non-active site disulfide of human Trx1**

According to the results of the above experiment, we further tested if GSH system acted as an electron donor for reduction of the non-active site disulfide of human Trx1. The physiological concentration of GSH is in the millimolar range in cells, so we used millimolar concentration of GSH in the activity assays. As shown in Fig. 4A, TrxR1 was unable to reduce the oxidized Trx1SGPS, in line with the previous report (14). However, physiological concentrations of GSH showed the ability to reduce the non-active site disulfide of Trx1SGPS (Fig. 4A) in the presence of glutathione reductase and NADPH. Further experiments (Fig. 4B) performed in the presence of human glutaredoxin 1 (Grx1) showed that this reduction was strongly enhanced by Grx1. We also performed the activity assay using Trx1-S₄ as the substrate and obtained the similar result (Fig. 4C). Because of the high background of GSH in the reduction of Trx1SGPS and Trx1-S₄, however, it was not possible to determine kinetic constants of this reaction. Therefore, the efficiency of Grx1 reducing Trx1SGPS and insulin was compared at the same concentration. As shown in Fig. 4B, Grx1 showed higher efficiency to reduce Trx1SGPS compared with insulin.

To explore the mechanism of Grx system reducing the non-active site disulfide of Trx1, the activity assay was performed with *E. coli* GrxC14S mutant protein, which contained only one cysteine. As shown in Fig. 5, *E. coli* GrxC14S showed the ability to reduce oxidized Trx1SGPS in the presence of GSH, glutathione reductase and NADPH, indicating that the reaction catalyzed by Grx1 followed the mono-thiol mechanism.

**DISCUSSION**

Trx1 plays important roles in redox regulation of signaling. All these roles that have been reported so far are dependent on the active site thiols of Trx1. In this study, we reported that the non-active site thiols of Trx1 also played important roles in redox regulation of cell signaling via reversible oxidation.

Although TrxR1 is responsible for reducing active site disulfide bridge of Trx1, TrxR1 is unable to reactivate the non-active site disulfide of Trx1. So it was interesting to know if the oxidation of the non-active site thiols was reversible or not in cells. In our study, it was suggested that Trx1 was protected by GSH
system from oxidation by ebselen in cells, as shown in Fig. 3. In addition, we also found that the glutaredoxin system worked as an electron donor for reduction of the two-disulfide form Trx1, which might allow oxidation of Trx1 in redox signaling to be reversible and regulatable under oxidative stress. The redox potential of the non-active site disulfide of human Trx1 is more positive than -210 mV, which is higher than that of the active site disulfide of human Trx1 with -230 mV (14). Because in vivo redox measurements in yeast and mammalian cells of GSH confirm a very reduced state for the cytosolic GSH/GSSG pool of -290 to -300 mV (29-31), the reaction is thermodynamically possible. It was reported previously that the glutaredoxin system also worked as an electron donor for reduction of the active site disulfide of human Trx1 (24). In a two-disulfide Trx1, the reduction of the active site disulfide might occur after the reduction of the non-active site disulfide, because the redox potential of the non-active site dithiol is higher than that of the active site. We proposed that the non-active site disulfide was reduced by GSH via a simple chemical reaction, as shown in Fig. 6A. Different from other protein-disulfide reductase, Grx1 also operated with a so-called mono-thiol mechanism besides the classic two-thiol mechanism (32). Based on our results in Fig. 5, we propose that the reduction of oxidized Trx1 by Grx1 system is a de-glutathionylation reaction through the mono-thiol mechanism, as illustrated in Fig. 6. In fact, the crosstalk between the Trx and Grx systems with the catalytic disulfide of some plant Trxs reduced by the Grx systems have been reported previously, such as the poplar Trx h4 (33,34), the Arabidopsis thaliana Trx h9 (35) and the grape Trx h (VvCxxS2) (36). According to the authors, the reduction of plant Trx by Grx system is a de-glutathionylation reaction (37), which is as same as the mechanism proposed in our study.

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Under oxidative stress, the inactivation of Trx1 via peroxides induced oxidation which could not be reversed by TrxR1 may provide time for the transmission of oxidative signals. As shown in our results, oxidative stress via short time treatment with H$_2$O$_2$ resulted in oxidation of cellular Trx1. It is very important to know the mechanism of the formation of the two-disulfide form Trx1 in cells under oxidative stress. We found that H$_2$O$_2$ alone was not an efficient oxidant for oxidation of Trx1. As a substrate of Prx1, H$_2$O$_2$ has more affinity to Prx1 than Trx1. Therefore, Prx1 was more susceptible to be oxidized by H$_2$O$_2$, and then Prx1 but not H$_2$O$_2$ oxidized Trx1 and gave rise to formation of the non-active site disulfide of Trx1. This might be similar to the case of Prx4, which was an endoplasmic reticulum resident protein with the capacity of coupling H$_2$O$_2$ catabolism with oxidative protein folding. It was proposed that Prx4 was first oxidized by H$_2$O$_2$ to form a disulfide, and then the disulfide of Prx4 was transferred to protein-disulfide isomerase thios for oxidation of newly synthesized proteins to be folded (20,38,39).

More and more evidences point out that H$_2$O$_2$ is primarily signaling messenger rather than evil oxidant (17,40,41). Many types of mammalian cells produce H$_2$O$_2$ for the purpose of intracellular signaling in response to stimulation through various cell surface receptors (17,42). H$_2$O$_2$ acts by oxidizing the critical residues of effectors to allow temporal inhibition of the effectors such as protein-tyrosine phosphatases (PTPs) (43) and the tumor suppressor PTEN (phosphatase and tensin homolog) (18). Presently, it is thought that the reversible inactivation of Prxs by H$_2$O$_2$ is involved in cell signaling. This is the called floodgate hypothesis. In this hypothesis, Prxs act as a peroxide floodgate, keeping peroxides away from susceptible targets until the floodgate is opened. Inactivation of Prxs occurs by overoxidation of the active site cysteine to sulfinic acid and this permits accumulated H$_2$O$_2$
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to then react with its targets (17,19,44). More recently, a report proposed that Prx2 was inactivated by hyperoxidation of its catalytic cysteine, while Prx1 was inactivated locally by phosphorylation but rather by hyperoxidation during H$_2$O$_2$ signaling at cell membrane (20,45). However, since Trx1 is an efficient electron donor to Prx1, Prx1 is not supposed to be inactive if Trx1 is still active. Therefore, we propose that inactivation of Trx1 is the mechanism to inactivate the Prx system which also supports the floodgate hypothesis. As shown in our results, H$_2$O$_2$ alone was not an efficient oxidant for oxidation of Trx1. But oxidation of Trx1 by H$_2$O$_2$ could be accelerated via Prx1 catalysis. Then the oxidized Trx1 was inactive as a substrate for TrxR1, resulting in the breakage of the electron chain from NADPH to Trx1 via TrxR1, and therefore the oxidized Trx1 was also inactive as a disulfide reductase. Eventually, this would lead to oxidation of Prx1 and local accumulation of H$_2$O$_2$ due to lack of electron donor, which subsequently resulted in inhibition of PTPs and PTEN.

In our previous study (24), we found that oxidation of Trx1 would lead to intracellular reactive oxygen species (ROS) accumulation and cell death. Nowadays, ROS have been shown to be function as signaling molecules (42). Carlos Gilter reported that the transient oxidation of Trx1 increased in parallel with H$_2$O$_2$ formation and the oxidized forms of several cellular proteins (46). Besides Prx system, ASK1, NF-κB, p53, Ref-1 and AP-1 pathways would also benefit from the inactivation of Trx1 (7,47). Fully reduced Trx1 can bind to ASK1 and inhibit its activity, whereas the oxidation of Trx1 results in the activation of ASK1 and the induction of ASK1-dependent apoptosis (48). The same is true for Txnip, that is, only reduced Trx1 binds to Txnip, which controls the activity of Trx system and is downregulated in tumor cells (49-51). In addition, Trx1 activity is essential to keep the proteins active by reducing critical Cys residues for activity of the transcription factor to bind to DNA, such as NF-κB, Ref-1 and AP-1.

In conclusion, reversible oxidation of Trx1 via the non-active site dithiol plays an important role in the transmission of oxidative signals and cellular redox signaling. To understand the precise mechanism of the non-active site cysteine residues of human Trx1 regulating the redox signaling under oxidative stress, further studies should be carried out. Especially challenging is to explore the role of the reversible inactivation and redox state of Trx1 in signaling pathway through the cell membrane.

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Trx1 is inactivated by Prx1 and reactivated by Grx system

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FIGURE LEGENDS

Figure 1. Redox state of Trx1 in A549 cells under oxidative stress. A, principle of redox Western blot analysis. To prepare mobility standards, cell lysates are denatured with urea and fully reduced with DTT. Then, varying molar ratios of IAA to IAM are incubated with the reduced Trx1 containing five cysteines, producing six protein isoforms with introduced number of acidic carboxymethyl thiol adducts (-SA) and neutral amidomethyl thiol adducts (-SM). During urea-PAGE, the ionized -SA group resulted in faster protein migration toward the anode. Therefore, the six isoforms are separated and used as a mobility standard for representing the number of -SA. To determine the redox state of Trx1 in cells, cells are lysed in urea lysis buffer containing IAM. After the free thiols of Trx1 were alkylated by IAM, cell lysates are precipitated by ice-cold acetone-HCl. The precipitate was washed with ice-cold acetone-HCl two more times to remove excess IAM. Then the precipitate was resuspended in urea lysis buffer containing DTT to reduce disulfides of Trx1. The free thiols of Trx1 were then alkylated by IAA. The alkylated Trx1 in cell lysates are separated according to the charge amount, representing the initial amount of free thiols of Trx1. B, A549 cells were exposed to oxidative stress (15 mM H2O2) for indicated time (lane 2-5), and then the redox state of Trx1 in A549
cells was detected with a redox western blot analysis. Lane 1, artificial mobility standards.

Figure 2. Redox state shift of human Trx1-S2 and Trx1SGPS in the presence of Prx1 and/or H₂O₂. Trx1-S2 was prepared as described in method section. 10 μM of Trx1-S2 or reduced Trx1SGPS was incubated with the indicated concentration of Prx1/H₂O₂ (lane 1-5) for 5 min at 37°C. Then the redox state of Trx1 and Trx1SGPS was detected with a redox urea-PAGE gel analysis. Lane 6, artificial mobility standards.

Figure 3. Redox state of Trx1 in HeLa cells treated with ebselen. HeLa cells were exposed to the indicated concentration of ebselen for 2 hours with or without 0.1 mM BSO pre-treatment (lane 2-7). Then the redox state of Trx1 in HeLa cells was detected with a redox western blot analysis. Lane 1, artificial mobility standards.

Figure 4. Reduction of oxidized Trx1SGPS and Trx1-S4 by GSH/Grx system. A, 45 nM glutathione reductase, 0.25 mM NADPH and 20 μM oxidized Trx1SGPS were added to cuvettes for GSH reduction assay in the presence of 3 (black solid line), 6 (dashed line) and 10 (dotted line) mM GSH. 0.25 mM NADPH and 20 μM oxidized Trx1SGPS were added to cuvettes for the TrxR1 reduction assay in the presence of 10 nM TrxR1 (gray solid line). The absorbance change at 340 nm was monitored. B, 45 nM glutathione reductase, 0.25 mM NADPH, 1 mM GSH and indicated amount of oxidized Trx1SGPS/insulin were added to cuvettes for reduction assay with or without 1 μM human Grx1. The absorbance change at 340 nm was monitored. C, 45 nM glutathione reductase, 0.25 mM NADPH, 1 mM GSH and indicated amount of oxidized wtTrx1 were added to cuvettes for reduction assay with or without 1 μM human Grx1. The absorbance change at 340 nm was monitored.

Figure 5. Reduction of oxidized Trx1SGPS by E. coli GrxC14S mutant protein. 45 nM glutathione reductase, 0.25 mM NADPH, 1 mM GSH and 20 (black solid line) or 40 (dashed line) μM oxidized Trx1SGPS were added to cuvettes for Grx reduction assay in the presence of 1 μM E. coli GrxC14S mutant protein. 0.25 mM NADPH and 20 μM oxidized Trx1SGPS were added to cuvettes for the TrxR1 reduction assay in the presence of 10 nM TrxR1 (gray solid line). The absorbance change at 340 nm was monitored.

Figure 6. Proposed mechanism of Trx1-S4 in redox regulation and oxidative stress. A, Proposed mechanism of reduction of oxidized Trx1 by GSH/Grx1. The non-active site disulfide is reduced by GSH via a simple chemical reaction. This reaction is stimulated by Grx1 through the mono-thiol mechanism. B, Oxidative signal is transmitted from H₂O₂ to Trx1-S4 via Prx1. Trx1-S4 is inactive and therefore results in Prx1 oxidation and H₂O₂ accumulation, which is involved in redox regulation including inhibition of PTPs and PTEN. ASK1, NF-κB, p53, Ref-1 and AP-1 pathways will also benefit from the inactivation of Trx1.
Table 1. Summary of the most likely forms of Trx1 for each band in redox western blot

| Bands | The most likely forms of Trx |
|-------|-----------------------------|
| 5     | Trx-(SH)$_5$                |
| 4     | (SH)$_4$-Trx-S-S-Trx-(SH)$_4$ Trx-(SH)$_4$S-protein$^a$ | Trx-(SH)$_4$S-SG$^b$ | Trx-(SH)$_4$S-NO$^c$ |
| 3     | Trx-(SH)$_3$S$_2$           |
| 2     | (SH)$_2$S$_2$-Trx-S-S-Trx-S$_2$(SH)$_2$ | Trx-(SH)$_2$S$_2$S-SG | Trx-(SH)$_2$S$_2$S-NO |
| 1     | Trx- S$_4$(SH)             |
| 0     | S$_4$-Trx-S-S-Trx-S$_4$   | Trx-S$_4$S-SG | Trx-S$_4$S-NO |

$^a$ Disulfide between Trx1 and some specific proteins, such as Txnip and Ask1

$^b$ Glutathionylation of Trx1

$^c$ Nitrosylation of Trx1

Trx1 is inactivated by Prx1 and reactivated by Grx system.
Trx1 is inactivated by Prx1 and reactivated by Grx system

Fig. 1

A

Urea/IAM

DTT

IAMS

IAA

SH amount

Preparation of mobility standards
Detection of redox state in cells

B

Time (min) M 0 2.5 5 10

SH amount
Trx1 is inactivated by Prx1 and reactivated by Grx system.
Trx1 is inactivated by Prx1 and reactivated by Grx system

Fig. 3

[Image of a gel electrophoresis experiment showing the effect of ebselen and BSO on SH amounts in different samples.]
Trx1 is inactivated by Prx1 and reactivated by Grx system

Fig. 4
Trx1 is inactivated by Prx1 and reactivated by Grx system

Fig. 5

-0.04 mM Trx1SGPS + GrxC14S
-0.02 mM Trx1SGPS + GrxC14S
-0.02 mM Trx1SGPS + TrxR1
Trx1 is inactivated by Prx1 and reactivated by Grx system.

Fig. 6

A

\[
\text{Trx1} \xrightarrow{	ext{S}} \text{Trx1} \xrightarrow{\text{S-SG}} \text{Trx1} \xrightarrow{\text{SH}} \text{Trx1} \xrightarrow{\text{SH} + \text{GSSG}} \\
\text{Trx1} \xrightarrow{\text{S-SG}} \text{Trx1} \xrightarrow{\text{Grx1-SH}} \text{Trx1} \xrightarrow{\text{SH}} \text{Grx1-S-SG} \xrightarrow{\text{GSH}} \text{Grx1-SH} + \text{GSSG}
\]

B

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Prx1-S}_2} \text{Prx1-(SH)_2} \xrightarrow{\text{Trx1-(SH)_S}} \text{Trx1-S}_2 \xrightarrow{\text{Trx1-S}_2} \text{Trx1-S}_2 \xrightarrow{\text{Trx1-S}_2 \text{ (inactive)}} \text{redox signaling}
\]

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Prx1-(SH)S}} \text{Trx1-(SH)_2} \xrightarrow{\text{Trx1-S}_2} \text{Trx1-S}_2 \xrightarrow{\text{local H}_2\text{O}_2 \text{ accumulation}}
\]

TRANSMISSION OF OXIDATIVE SIGNAL
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