A Major Peroxiredoxin-induced Activation of Yap1 Transcription Factor Is Mediated by Reduction-sensitive Disulfide Bonds and Reveals a Low Level of Transcriptional Activation

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Redox reactions involving cysteine thiol-disulfide exchange are crucial for the intracellular monitoring of hydrogen peroxide (H$_2$O$_2$). Yap1, the master transcription factor for the oxidative stress response in budding yeast, is activated by the formation of disulfide bonds in response to H$_2$O$_2$. Gpx3 (glutathione peroxidase-like protein 3) acts as a receptor for H$_2$O$_2$, and Ybp1 (Yap1-binding protein 1) is crucial for Gpx3-dependent disulfide bond formation in Yap1. We previously reported that Tsa1, a major peroxiredoxin in yeast cells, is required for activation of Yap1 in a widely used yeast strain, W303-1b, carrying the ybp1-1 mutant allele encoding a truncated Ybp1 protein. In the present study, we show that Tsa1 can interact with Yap1 via disulfide linkages and induce the formation of intramolecular disulfide bonds in Yap1 in ybp1-1 cells. The results provide evidence that Prx can have intrinsic activity as an H$_2$O$_2$ receptor and can relay H$_2$O$_2$ as a signal to the Prx target proteins in terms of formation of disulfide linkage. Furthermore, our data reveal that there is more of the reduction-resistant active form of Yap1 (i.e. Yap1 (oxII)) when it is partnered with Gpx3 than with Tsa1. These data support our hypothesis that changes in the redox status of Yap1 to reduction-resistant forms by multiple disulfide bond formation are important for determining the level and duration of Yap1 activity in the dynamic equilibrium of redox reactions in cells exposed to H$_2$O$_2$.

Oxygen serves as an electron acceptor for the efficient production of ATP. However, oxygen can also be converted to toxic reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and superoxide, which can damage cellular macromolecules (1). ROS can also act as a cellular signal in response to growth factors, cytokines, or other extracellular signals (2). Thus, organisms have acquired systems for sensing levels of ROS, not only to protect cells from oxidative stress but also to transmit ROS to other molecules as a signal. Superoxide is converted to H$_2$O$_2$ by the abundant protein superoxide dismutase. Thus, sensing of H$_2$O$_2$ is an important step in ROS signaling (3).

In bacteria, sensing of H$_2$O$_2$ involves transcription factor OxyR (4–6), whereas transcription factors Yap1 and Pap1 fulfill this role in yeasts. In response to H$_2$O$_2$, these transcription factors activate the transcription of genes for enzymes that mitigate oxidative stress. OxyR reacts directly with H$_2$O$_2$ to form sulfenic acid at Cys-199, which rapidly forms a disulfide bond with Cys-208, resulting in activation of the expression of the target genes. OxyR is rapidly deactivated by reduction of the disulfide bond by a glutathione-dependent mechanism (4, 5, 7, 8). In contrast, peroxiredoxin activity is required for activation of Yap1 and Pap1 (see below).

Peroxiredoxin (Prx) is a ubiquitous peroxidase family found in species ranging from *Escherichia coli* to humans (9). In budding yeast, proteins that show thioredoxin (Trx)-dependent peroxidase activity include five Prx family proteins (10) and two glutathione peroxidase (Gpx)-like proteins (11, 12) (Fig. 1A). Prx (we will use Prx here to refer to the proteins that include Prx and the Gpx-like proteins) can reduce H$_2$O$_2$ at the expense of electrons from NADPH through the Trx-dependent redox system (Fig. 1B). In fission yeast, Tpx1 and Gpx1 reveal Trx-dependent peroxidase activity. Gpx1 serves to ensure optimal mitochondrial function and cytosolic environment, especially in the late growth phase (stationary phase) (13). The catalytic Cys residue of Prx is directly oxidized by H$_2$O$_2$. This oxidation is followed by the formation of a disulfide bond linkage with a resolving Cys of the same molecule of Prx (Fig. 1C) or with a resolving Cys of another Prx molecule (dimer formation).

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5 The abbreviations used are: ROS, reactive oxygen species; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; c-CRD, carboxyl-terminal cysteine-rich domain; DTT, dithiothreitol; Gpx, glutathione peroxidase; HA, hemagglutinin; n-CRD, amino terminal cysteine-rich domain; NEM, N-ethylmaleimide; Prx, peroxiredoxin; Trx, thioredoxin; SD, synthetic dextrose; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethy)glycine.
Recent findings on the sensing and transduction of H₂O₂ in budding and fission yeasts suggest that Prx can function as a receptor for hydroperoxide and that the transcription factor Yap1 (budding yeast) (11) and its orthologue Pap1 (fission yeast) (14, 15) can act as sensors/transducers of the H₂O₂ signal. In budding yeast, Gpx3 (also known as Orp1) is involved in Yap1 activation (11), whereas Tpx1 is involved in Pap1 activation (14, 15). A precise mechanism for the activation of Yap1 has been uncovered. Yap1 is mainly localized in the cytoplasm under nonstress conditions. The cytoplasmic localization is determined by constitutive nuclear export that predominates constitutive nuclear import (16, 17). In response to H₂O₂ stress, a disulfide bond is formed in Yap1, and the conformation of its nuclear export signal, which is embedded in the carboxyl-terminal cysteine rich domain (c-CRD; see Fig. 2A), is altered (16–18). The formation of the disulfide bond is catalyzed by Gpx3 (Fig. 1C). A catalytic cysteine of Gpx3 is directly oxidized by H₂O₂. This oxidation is followed by the transient formation of a disulfide bond with Cys-598 in the c-CRD. A thiol-disulfide exchange reaction results in intramolecular formation of a disulfide bond between the c-CRD and the amino-terminal cysteine-rich domain (n-CRD) (19). Oxidized Yap1 (20) and oxidized Gpx3 (11) are reduced at the expense of electrons from NADPH through the Trx-dependent redox system (Fig. 1B). Our recent finding (21) suggested that the formation of multiple disulfide bonds between two cysteine-rich domains of Yap1 was responsible for altering the redox state of Yap1. This formation results in an increase in the oxidized (active) form of Yap1 in the dynamic equilibrium of both oxidation and reduction reactions of Yap1.

In addition to Gpx3, Ybp1 (Yap1-binding protein 1) is required for Gpx3-induced activation of Yap1 in response to H₂O₂ (22). A widely used laboratory strain, W303-1b (22–26), contains an allele of ybp1-1 that has a “C” insertion at position 729, which generates a termination codon at amino acid position 243 (27). We reported previously that activation of Yap1 is solely dependent on Tsa1 in ybp1-1 (initially called ybp1-2) cells and that ybp1-1 cells are more sensitive to H₂O₂ than wild-type YBP1 cells (order of sensitivity: YBP1 < ybp1-1 < ybp1Δ < YBP1 yap1Δ) (27). Because the steady state protein level of Tsa1 is 45 times that of Gpx3 (28) (Fig. 1A), it is possible that the transient interaction of Tsa1 with Yap1 may facilitate H₂O₂-induced disulfide bond formation in Yap1 in the context of a ybp1-1 genetic background. Thus, study of the mechanism of Tsa1-dependent activation of Yap1 in W303-1b in response to H₂O₂ may provide evidence that indicates that the Prx family can intrinsically serve as a receptor for H₂O₂ and other hydroperoxides and can relay hydroperoxides as a signal to the Prx target proteins in terms of the formation of disulfide linkage.

In the present study, we addressed the question of whether Tsa1 could be responsible for the formation of the disulfide bond in Yap1 in ybp1-1 cells in response to H₂O₂. We demonstrate that Tsa1 can interact with Yap1 through a disulfide linkage and can induce the formation of intramolecular disulfide bonds in Yap1. We also suggest that the Tsa1-induced formation of disulfide bonds is not sufficient to shift the redox state of Yap1. These results explain the mechanism for the H₂O₂-sensitive phenotype of ybp1-1 cells and for the lower transcriptional activity of Yap1 in ybp1-1 cells compared with YBP1 cells (22, 27).

**EXPERIMENTAL PROCEDURES**

**Media and Strains**—Yeast cells were grown in synthetic dextrose (SD) medium supplemented with amino acids (SD drop-out) (29) or in YPAD medium (1% peptone, 0.5% yeast extract, 2% glucose, 0.08 mg/ml adenine sulfate) at 30 °C. The following strains of *Saccharomyces cerevisiae* were used as ybp1-1 cells in this study: Y700 (W303-1b; *MATa his3 can1-100 ade2 leu2 trp1 ura3 ybp1-1) (27), TW (the same as Y700 but *tsa1::URA3 UR3A::TRX2p::LacZ*) (26), and TW *tsa1::HIS3* (the same as TW but *tsa1::HIS3*) (27). The ybp1-1 allele was first reported by Veal et al. (22). Initially, we designated the YBP1 allele of Y700 as ybp1-2, because we had found a base insertion at nucleotide position 729 that produced a nonsense mutation at amino acid position 244 (nucleotide position 730) (27) in addition to the I7L substitution found in ybp1-1. However, Veal et al. (22) corrected their sequence data for ybp1-1 and ybp1-2 is the same.

**FIGURE 1. Steady state level of Prx in budding yeast cells.**

A, cellular Prx family proteins in *S. cerevisiae*. Proteins that have Trx-dependent peroxiredoxin activity include five homologous Prx proteins and two Gpx-like proteins. The numbers of molecules per cell (28) are indicated by the bar, and the percentages of the total are indicated above the bar. Levels of Yap1 and Ybp1 are also shown. The number of molecules of Gpx3 per cell is more than 45 times greater than that of Tsa1. B, Prx coupled with Trx, thioredoxin reductase (Trn), and NADPH can reduce H₂O₂ using a high energy electron from NADPH. C, the formation of the disulfide bond is catalyzed by Gpx3 (see “Results”). The peroxiredoxin activity of Gpx3 is also indicated.
**Tsa1-induced Disulfide Bond in Yap1 in ybp1-1 Cells**

We used Y17202 (MATα hisΔ1 leu2Δ0 lys2Δ0 trp1 ura3Δ0 tpi1::kanMX4), namely trp1Δ cells from a knock-out library constructed using BY4742 (EUROSCARF, Frankfurt, Germany) derived from S288C and BY4742 yap1Δ (MATα hisΔ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1::kanMX4 yap1::URA3).

### Analysis of the Redox Status of Yap1 in Yeast Cells

Lysates of yeast cells that expressed the hemagglutinin epitope HA-tagged regulatory domain of Yap1 (amino acid residues 122–650, HA3-Yap1) were prepared essentially as described previously (19) with slight modifications (21). Briefly, yeast cells treated with H₂O₂ were suspended in 10% trichloroacetic acid (TCA) at a final concentration of 50 mM. The mixtures were then dialyzed extensively against a buffer containing 10 mM Tris-HCl (pH 8), 50 mM NaCl, and 10 mM MgCl₂. Samples were treated with calf intestinal alkaline phosphatase, mixed with one-third volume of 3 × SDS-sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, and 0.03% bromophenol blue), and fractionated by electrophoresis on a 7.5–17.5% gradient polyacrylamide gel (ratio of acrylamide to bisacrylamide, 30:4). We performed the immunoblotting and detection of HA-tagged Yap1 as described previously (21). We obtained chemiluminescent images of the blotted membrane using ECL (GE Healthcare) and Lumi-Film chemiluminescent detection film (Roche Applied Science).

### Kinetics-based Redox Analysis of Recombinant Proteins

- All reagents used for redox analysis were degassed, and experiments were performed under argon or nitrogen.
- Redox cycles of the regulatory domain of Yap1 (amino acid residues 122–650), n-CRD, and c-CRD in the presence of Tsa1, Trx, Trr, and NADPH were studied as described previously. Briefly, E. coli BL21 (DE3) cells carrying expression vectors were grown in L-broth at 30 °C, lysed, and purified on GSH-Sepharose (GE Healthcare) as previously described (21, 26).
- The glutathione S-transferase fused Yap1 proteins (the Yap1 regulatory region (amino acid residues 122–650) and its mutants, n-CRD (amino acid residues 122–373) and c-CRD (amino acid residues 578–650)) were released by cleavage of glutathione S-transferase from each protein using PreScission Protease™ (GE Healthcare). Polystyrene-tagged (His-tagged) proteins (Tsa1, Trx2, and Trr1) were purified by two cycles of chromatography on a column of Ni²⁺-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany).

Proteins were quantified by Bradford’s method with bovine serum albumin as the standard. A reaction mixture containing 55.2 µM Tsa1, 11.8 µM Trx2, and 3.9 µM Trr1 was prepared in reaction buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.33 mM NADPH) and incubated at room temperature for 30 min, after which 10.5 µM Yap1 and 44.7 µM c-CRD were added. The oxidizing reaction was started by the addition of 0.4 mM H₂O₂. Aliquots were removed at the indicated times and mixed with AMS at a final concentration of 10 mM at 30 °C for 30 min. We then fractionated the proteins by SDS-PAGE (15% polyacrylamide or a gradient of 7.5–17.5% polyacrylamide; ratio of acrylamide to bisacrylamide, 30:0.4) in a running buffer that contained 25 mM Tris, 250 mM glycerine, and 0.1% SDS. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma).

**Immunoprecipitation and β-Galactosidase Assay**

- To create the expression plasmid for Tsa1/C171T, Cys-171 was mutagenized by PCR with a mutant oligonucleotide of the TSA1 open reading frame (TGTA, a codon at 171, was mutated to ACT) that had been cloned into pET-15b. Then we cloned the HIS-tag-TSA1C171T region with the Tsa1 promoter region into pRS315 and designated the resulting plasmid as pRS-315-HIS-TSA1/C171T. pRS-315-HIS-tag-TSA1C171T was constructed similarly. We treated a log phase culture (A₆₀₀ = 0.3) of TW tsa1::HIS3 cells carrying both pRS314-cup-HA-tag-Yap1-(122–650) (21) and pRS-315-HIS-tag-TSA1C171T in SD dropout (−Leu, −Trp) with 0.15 mM CuSO₄ for 2 h to induce the production of HA-tagged Yap1-(122–650) protein. We changed the medium to fresh SD dropout (−Leu, −Trp) containing 0.4 mM H₂O₂ and cultured the cells for a further 5 min. We washed the cells with 10 mM N-ethylmaleimide (NEM) and suspended them in Buffer L (100 mM Tris-HCl (pH 8), 50 mM NaCl, 0.2% deoxycholate, 0.15% Nonidet P-40, 50 mM NEM, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM N-p-tosyl-l-phenylalanine chloromethyl ketone, 0.05 mM N°-tosyl-L-lysine chloromethyl ketone hydrochloride, 2 µg/ml pepstatin A, 5 µg/ml aprotinin, 3 µg/ml leupeptin, and 1 mM EDTA). Cells were then frozen in liquid nitrogen and disrupted by grinding in a mortar under dry ice. We collected the supernatant of the lystate after centrifugation (15,000 × g, 10 min) and performed immunoprecipitation on monoclonal anti-HA-agarose conjugate (Sigma) that had been swollen in Buffer W1 (same as Buffer L but without NEM) containing 0.1% skimmed milk. We washed the conjugate with Buffer W1 eight times and with Buffer W2 (100 mM Tris-HCl (pH 8), 50 mM NaCl, 0.2% deoxycholate, 0.15% Nonidet P-40) two times. After boiling the immunoprecipitate in the SDS-sample buffer with or without 10 mM dithiothreitol (DTT), we fractionated the proteins by SDS-PAGE. We obtained chemiluminescent images of the filter using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA) and the Varsa-Doc™ imaging system (Bio-Rad).

The assay of β-galactosidase activity was performed as described in Ref. 21, except that we used 4-methylumbel-feriliferyl β-D-galactopyranoside (Sigma) as the substrate. We determined the fluorescence (450 nm) of 4-methylumbiferiferon obtained by excitation at 360 nm to estimate activity.

**Determination of the Reduction Potential of Yap1 and Its Domains**

- We performed a redox titration of NADPH in the Trx/Trr/NADPH redox system following the procedure described in Ref. 21. The reaction mixture contained 22.5 µM Tsa1, 4.82 µM Trx2, 1.62 µM Trr1, and 0.4 mM NADPH and was incubated at room temperature for 30 min to reduce the entire system. After the addition of a 10.5 µM concentration of the regulatory domain of Yap1, 22.5 µM n-CRD, and 44.6 µM c-CRD to the reaction mixture, H₂O₂ was added to a final concentration of 0.4 mM, and the incubation was continued for 30 min, by which time the reaction had reached equilibrium. The...
samples were then divided into tubes that contained various amounts of NADPH (final concentrations of 0.44, 0.48, 0.52, 0.56, 0.60, and 0.64 mM), and the samples were incubated for another 1 h until they again reached equilibrium. The redox reactions were stopped by the addition of AMS, and the reduced and oxidized forms of each protein were quantified after SDS-PAGE. For separation of the Yap1 domains, Tsa1, and Trx2, we used an SDS-polyacrylamide gel (15% polyacrylamide; ratio of acrylamide to bisacrylamide of 29:1) in a Tris-Tricine buffer system (Daichii Pure Chemicals, Tokyo, Japan) under nonreducing conditions.

Reproducibility—All of the experiments were repeated at least three times, and representative results are shown.

RESULTS

Oxidation of Yap1 in ybp1-1 Cells—We showed previously that the transcriptional activation of Yap1 in response to H$_2$O$_2$ is dependent on Tsa1 but not on Gpx3 in cells with the ybp1-1 genetic background (27). Yap1 has three cysteines in the n-CRD and three in the c-CRD. The six cysteines have an important function in the H$_2$O$_2$-dependent response (19, 21), whereas only the three cysteines in the c-CRD are required for the response to thiol oxidants and electrophilic reagents (24, 30). Because mutation of the three Cys residues in the n-CRD (Yap1$^{TTT,CCC}$) decreased the H$_2$O$_2$ resistance of Y700 (ybp1-1) cells (data not shown), the disulfide bond(s) between the n-CRD and c-CRD may be crucial for activation of Yap1 in response to H$_2$O$_2$ in ybp1-1 cells.

We investigated the redox status of the cysteine residues (free thiols or disulfide bonds) of Yap1 based on two criteria (21). First, free cysteine residues were detected in terms of increasing molecular weight by reacting the free thiol group with the thiol-alkylating reagent AMS (0.5 kDa). Second, due to conformational constraints of the SDS-denatured polypeptide chain of Yap1 (19), the formation of intramolecular disulfide bonds between the n-CRD and c-CRD resulted in enhanced mobility, which was detected by SDS-PAGE. We designated this form Yap1 (oxII) (Fig. 2A).

As previously reported (21), the amount of AMS that reacts with Yap1 decreased within 10 s after treatment of YBP1 cells with H$_2$O$_2$ (Fig. 2B). This Yap1 corresponds to an oxidized Yap1 with one disulfide bond formed within the n-CRD (Yap1 (oxI)). Yap1 (oxII) appeared later (30–300 s) as Yap1 (oxI) decreased. In ybp1-1 cells, a faster migrating Yap1 with mobility corresponding to that of Yap1 (oxI) also appeared within 10–900 s (designated as Yap1 (oxI-)) (Fig. 2C). As controls, we detected Yap1 mutant proteins in cells expressing the Yap1$^{TTT,TAT}$ mutant, in which all six Cys residues were mutated and should not react with AMS, and in cells expressing the Yap1$^{TTT,CCC}$ mutant, in which only three Cys residues in the c-CRD remained (Fig. 2A). There were no significant differences in mobility between Yap1$^{TTT,TAT}$ (treated with AMS but not alkylated due to lack of Cys residues) and the AMS-alkylated form of reduced-Yap1$^{TTT,CCC}$ (Fig. 2B). This suggested that most of the AMS-reactive Cys residues in Yap1 might be in the n-CRD. Thus, these results suggested that, upon exposure to H$_2$O$_2$, a possible disulfide bond of Yap1 (oxI-)) in ybp1-1 cells might be formed within the n-CRD. Therefore, disulfide bond formation in Yap1 (oxI-)) might be similar to that in Yap1 (oxI) (see Fig. 2A). Consistent with a previous report (22), the level of the active form of Yap1 (Yap1 (oxII-)) was significantly lower in ybp1-1 cells than in YBP1 cells (Fig. 2B), although the process of disulfide bond formation in Yap1 was similar in both cell types.

Time-dependent Transcriptional Activation of Yap1 in ybp1-1 Cells—A disulfide bond must be formed between the n-CRD and c-CRD for inhibition of the interaction between the nuclear export receptor Crm1 and the nuclear export signal (NES) that is embedded in the c-CRD (16, 19, 31). Thus, the levels of nuclear localized Yap1 and Yap1-dependent transcriptional activation are correlated with the formation of Yap1 (oxII) in cells exposed to H$_2$O$_2$. We showed a time-dependent activation of the Yap1 target gene TRX2 encoding Trx in both YBP1 and ybp1-1 cells. As shown in Fig. 3, transcriptional activation in

![Figure 2](image.jpg)

**Yap1 is oxidized to two different forms in ybp1-1 cells.** A, six Cys residues with the residue numbers in Yap1 in the two Cys-rich domains (n-CRD and c-CRD) and the nuclear export signal (NES)-containing region are indicated. Gpx3-induced oxidized forms of Yap1 with bars linking two Cys residues are indicated. Yap1 Cys mutants used in $B$ are indicated. Control experiments were treated with AMS. An anti-HA antibody was used for immunoblotting to detect Yap1. Forms of oxidized Yap1 (Yap1 (oxI) and Yap1 (oxII)) and reduced Yap1 (Yap1 (red)) are indicated. $C$, Yap1 is oxidized to two different forms in ybp1-1 cells. Y700 ybp1-1 cells expressing an HA$_3$-tagged version of full-length Yap1 were treated with H$_2$O$_2$ for the indicated time (s). A high contrast picture is also shown.
YBP1 cells started about 10 min after the exposure of H2O2, and a maximal level of activation was observed at 40 min. In contrast, the activation started at 40 min in ybp1-1 cells, and the activation was extended in ybp1-1 cells (100 min). Overall levels of transcript are higher in YBP1 than in ybp1-1 cells. The levels of transcription during the first 15 min after exposure to H2O2 seemed to be correlated with the level of formation of Yap1 (oxII-T) (see Fig. 2C and the previous report (22)).

Both the Catalytic and the Resolving Cys of Tsa1 Are Required for the Activation of Yap1—To introduce the disulfide bond formation in Yap1, the catalytic Cys (Cys-56) of Gpx3 but not the resolving Cys is required (11). We examined the requirement of such Cys residues of Tsa1 by the reporter gene assay. As shown in Fig. 4, both catalytic Cys-48 and resolving Cys-171 were required for Tsa1-dependent activation of Yap1 in ybp1-1 cells.

Cys-48 of the Tsa1C171T Mutant Can Form Mixed Disulfide Bonds with Cys Residues of Yap1—Introduction of a disulfide bond in Yap1 via Gpx3 is postulated (11) to occur as follows. The catalytic cysteine (Cys-56) of Gpx3 is first oxidized to sulfinic acid by H2O2. This can then form a transient disulfide bond with Yap1 Cys-598, and finally, the thionilate anion of Yap1 Cys-303 attacks the disulfide bond to form Cys-303–Cys-598 in Yap1 to form Yap1 (oxII) (see Fig. 1).

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which the resolving Cys at 171 was mutated, formed multiple complexes with Yap1 within s o f the addition of H2O2. The slower migrating Yap1-Tsa1C171T proteins (Complex in Fig. 5A) were fractionated in 6% polyacrylamide gels (SDS-PAGE; Fig. 5B and C), and the molecular weights were estimated as 98,000, 132,000, 160,000, and 191,000, which may be equivalent to Yap1 (68,000; the regulatory domain) linked to one to four molecules of Tsa1C171T, respectively. In fact, immuno- blot analysis using the anti-Tsa1 antibody suggested that this band of proteins contained the Tsa1 protein (Fig. 5A). Because the complexes were sensitive to DTT treatment (Fig. 5A, DTT), they might consist of multiple Tsa1 molecules linked via disulfide bonds between Cys-48 of Tsa1 and more than one Cys of Yap1.

Next, we examined the formation of a complex between Yap1 and Tsa1C171T in yeast cells. We immunoprecipitated HA-Yap1 from cell lysates of ybp1-1 cells expressing both HA-tagged Yap1 and Tsa1. Lysates were prepared from cells treated with trichloroacetic acid in the presence of a thiol-alkylating agent to prevent dithioldisulfide exchange during immunoprecipitation. After the immunoprecipitates were subjected to SDS-PAGE, we detected Tsa1 by immuno blotting with an anti-Tsa1 antibody. As shown in Fig. 6A, four slower migrating bands of protein were observed in the pull-down fraction in the lysate from the cells expressing Tsa1C171T (lane 5). Similarly, a slower migrating band of protein was detected in the pull-down fraction from the lysate of cells expressing wild type Tsa1 (lane 3), although the level of the complex was lower than that observed in lane 5. Additionally, most Tsa1 in lane 3 migrated at a molecular weight of 50,000, which corresponded to the dimer form of Tsa1. As shown in Fig. 6B, levels of wild type Tsa1 attached to Yap1 in the pull-down fraction, which was examined after reduction of disulfide bond, were smaller than that of Tsa1C171T attached to Yap1 (compare lanes 3 and 5). These results suggested that Cys-48 of Tsa1 might form transient disulfide bonds with Yap1 Cys residues in the cells exposed to H2O2 and that the mixed disulfide formation of Yap1-Tsa1 might be stabilized when the resolving Cys (Cys-171) of Tsa1 was mutated.

Oxidation (Disulfide Bond Formation) Process of Yap1 in the in Vitro Trx Redox System—Next, we verified the process of disulfide bond formation in Yap1 in vitro. We previously reported the process of Gpx3-dependent disulfide bond formation in Yap1 using a reconstituted in vitro redox system (21). Continuous oxidation of Gpx3 with H2O2 and continuous
Tsa1-induced Disulfide Bond in Yap1 in ybp1-1 Cells

A

B

C

D

Thermodynamic relationships between Yap1, Tsa1, and Trx2. A–C, oxidized Yap1, n-CRD, and c-CRD were titrated with various concentrations of NADPH in the presence of the Tsa1/Trx/Trr/NADPH system as shown in A and as described previously (21) and under "Experimental Procedures." We fractionated the reaction mixture in 7.5–17.5% polyacrylamide (B) and 15% polyacrylamide (C). The mobilities of the molecular weight markers are indicated by the molecular weight × 10⁻³. The asterisks in B and C indicate non-Yap1 proteins that were co-purified with recombinant proteins from lysates of E. coli. The positions of these oxidized and reduced proteins were determined by the previous study (21). The arrowheads indicate lanes or the middle of lanes in which the ratios of oxidized and reduced forms of each Yap1 (oxII-T), Tsa1, and Trx2 are approximately the same. D, the relative levels of oxidized Yap1 (oxII-T), Tsa1, and Trx2 are correlated with increasing levels of NADPH and decreasing levels of H₂O₂, which are represented by the width of the open block and the half-tone block, respectively. The predicted midpoint reduction potentials are shown. We determined the oxidized and reduced forms of Yap1 and Tsa1 and the oxidized form of Trx2 to estimate the ratios of the oxidized form to the reduced form. Using the reduction potential of Trx2 (~262 mV, pH 7.0, 25 °C), which was estimated by titration of lipoate redox buffer as described previously (21), we calculated the midpoint reduction potentials of Yap1 (oxII-T) as ~255 mV (an average of the value estimated by triplicate experiments; ~256, ~260, and ~250 mV) and of Tsa1 as ~211 mV (same as above; ~217, ~209, and ~211 mV). Note that the estimated reduction potential values of the n-CRD and c-CRD were variable. The Cys residues responsible for the disulfide linkage between the n-CRD and c-CRD of Yap1 (oxII-T) are unknown.

The in vitro redox system, including Tsa1, in response to the addition of H₂O₂ (21). After bacterially produced Yap1 was reduced in the Trx reduction system containing Tsa1, we treated the reaction mix with H₂O₂. As shown in Fig. 7A, most Yap1 (red) was oxidized to Yap1 (oxI-T) and Yap1 (oxII-T) within 1 min, and then Yap1 (oxI-T) was converted to Yap1 (oxII-T) within 5 min. Although Tsa1 (Fig. 7A) and Trx were fully oxidized within 1 min (data not shown), the fact that more than 70% of the NADPH still remained and the level declined during the next 20 min in this system (21) suggested that the continuous oxidation and reduction of the proteins (Yap1, Tsa1, and Trx2) yielded Yap1 (oxI-T) and Yap1 (oxII-T). As shown in Fig. 7B, the oxidation process was delayed significantly without the addition of Tsa1. These results suggested that, similar to Gpx3, Tsa1 might act as a catalyst in the formation of disulfide bonds in Yap1.

Reduction Potential of Yap1 (oxII-T)—Although the Tsa1-catalyzed oxidation of Yap1 in vitro (Fig. 7A) was similar to the Gpx3-dependent process (21), the level of the oxII form of Yap1 and the transcriptional level of the target gene of Yap1 were significantly lower in ybp1-1 cells. Thus, we next questioned whether qualitative differences between Yap1 (oxII-T) and Gpx3-catalyzed Yap1 (oxII) might cause these differences. Our previous study (21) suggested that the redox state of Yap1 had shifted to a more reduction-resistant form, depending on the number of disulfide bonds between the n-CRD and c-CRD. In fact, the shift in the redox state of Yap1 is crucial for the duration and level of activated (nuclear localized) Yap1. Thus, we reasoned that the reduction potential of Yap1 (oxII-T) might differ from that of

Reduction of oxidized Gpx3 by the Trx reduction system (Trx/Trr/NADPH) are required for Gpx3-dependent catalysis of the disulfide bond formation of Yap1. This might also be true in the case of Tsa1-dependent oxidation of Yap1, because disulfide-bonded Tsa1 failed to induce a disulfide bond in Yap1 (data not shown). We examined the time-dependent oxidation of Yap1 in

FIGURE 8. Thermodynamic relationships between Yap1, Tsa1, and Trx2. A–C, oxidized Yap1, n-CRD, and c-CRD were titrated with various concentrations of NADPH in the presence of the Tsa1/Trx/Trr/NADPH system as shown in A and as described previously (21) and under "Experimental Procedures." We fractionated the reaction mixture in 7.5–17.5% polyacrylamide (B) and 15% polyacrylamide (C). The mobilities of the molecular weight markers are indicated by the molecular weight × 10⁻³. The asterisks in B and C indicate non-Yap1 proteins that were co-purified with recombinant proteins from lysates of E. coli. The positions of these oxidized and reduced proteins were determined by the previous study (21). The arrowheads indicate lanes or the middle of lanes in which the ratios of oxidized and reduced forms of each Yap1 (oxII-T), Tsa1, and Trx2 are approximately the same. D, the relative levels of oxidized Yap1 (oxII-T), Tsa1, and Trx2 are correlated with increasing levels of NADPH and decreasing levels of H₂O₂, which are represented by the width of the open block and the half-tone block, respectively. The predicted midpoint reduction potentials are shown. We determined the oxidized and reduced forms of Yap1 and Tsa1 and the oxidized form of Trx2 to estimate the ratios of the oxidized form to the reduced form. Using the reduction potential of Trx2 (~262 mV, pH 7.0, 25 °C), which was estimated by titration of lipoate redox buffer as described previously (21), we calculated the midpoint reduction potentials of Yap1 (oxII-T) as ~255 mV (an average of the value estimated by triplicate experiments; ~256, ~260, and ~250 mV) and of Tsa1 as ~211 mV (same as above; ~217, ~209, and ~211 mV). Note that the estimated reduction potential values of the n-CRD and c-CRD were variable. The Cys residues responsible for the disulfide linkage between the n-CRD and c-CRD of Yap1 (oxII-T) are unknown.

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Tsa1 makes up 91% of the Prx protein in S. cerevisiae (Fig. 1A). In contrast, one of the low level Prx proteins, Gpx3, can act as an H₂O₂ receptor for Yap1 with the benefit of Ybp1 (22). Here, we showed that Tsa1 could induce the formation of multiple disulfide bonds in Yap1 in cells and in the reconstituted in vitro redox system. Further, we showed that disulfide linkages are formed between Yap1 and Cys-48 of Tsa1C¹⁷¹T, both in cells (Fig. 6) and in vitro (Fig. 5). These results suggested a model that is analogous to Gpx3-induced disulfide bond formation in Yap1. After Cys-48 of Tsa1 is oxidized to Tsa1-SOH by H₂O₂, the Tsa1-Yap1 complex is linked by a disulfide bond, and the following exchange reaction then produces intramolecular disulfide bonds (Fig. 9A). Our results indicated that, in addition to Gpx3, the major Prx family member may also serve as a receptor for H₂O₂ or other hydroperoxides and may relay it as a signal to possible individual target proteins.

The resolving Cys (Cys-171) of Tsa1 was required for Tsa1-induced activation of Yap1 (Fig. 4), whereas the resolving Cys of Gpx3 is not required (11). We previously reported that about one-third of Tsa1 forms homodimer linked via disulfide bond at the steady state (21). The level of the dimer Tsa1 slightly increases in response to H₂O₂. Thus, it is possible that the Cys-48-SH of the homodimer Tsa1 might be responsible for the disulfide bond formation in Yap1 (See Fig. 9A). This idea is supported by the finding that the Tsa1 homodimer was easily detached from Yap1 during immune precipitation, whereas Tsa1C¹⁷¹T was stably attached with Yap1 via disulfide bonds (Fig. 6).

We also addressed the redox state of the Tsa1-induced oxidation product of Yap1. Interestingly, the midpoint reduction potential of Yap1 (oxII-T) was higher than that of Gpx3-induced Yap1 (oxII-2), although the rate of the oxidation process of Yap1 induced by Tsa1 (Fig. 7) was similar to that induced by Gpx3 (21). Thus, the disulfide bond(s) formed between the n-CRD and c-CRD of Yap1 (oxII-T) might be inappropriate and was insufficient to maintain the oxII (active) form of Yap1 due to reduction by Trx (Fig. 2B and Fig. 9). This is consistent with the result indicating that the transcriptional activity driven by Yap1 (oxII-T) was increased during the late stage of H₂O₂ response (Fig. 3), when the reduction capacity (such as levels of NADPH and reduced Trx) might decline (21). The mechanism underlying the formation of reduction sensitive Yap1 (oxII-T) is unknown. Tsa1 forms multimers, such as dimers and decamers (33), whereas Gpx3 consists of monomers. Thus, it is possible that a Tsa1 multimer might interact with more than one Cys residue of Yap1 to form multiple disulfide bonds and to interrupt proper disulfide bond formation.

The catalytic Cys (Cys-48) of Tsa1 is hyperoxidized to sulfenic acid (-SO₂H) or sulfonic acid (-SO₃H) in the presence of H₂O₂ (34). We showed here that the midpoint reduction potential of Tsa1 was ~211 mV (Fig. 8), which is higher than that of Gpx3 (~254 mV). The higher midpoint reduction potential of Tsa1 might contribute to the hyperoxidization of the Cys-48 of Tsa1.

In contrast with budding yeast, oxidative stress sensing and transduction systems in fission yeast are concentration-specific and are integrated with two different activators/transcription factors, Tpx1/Pap1 (14, 15) and Sty1/ATF4 (35, 36). Tpx1-induced oxidation of Pap1 is carried out at lower levels of H₂O₂, whereas the mitogen-activated protein kinase (Sty1)-induced oxidation of Yap1 is carried out at higher levels of H₂O₂ (34). We showed here that the midpoint reduction potential of Tsa1 was higher than that of Yap1 (oxII-T) (21); see Fig. 8D). Because the estimated value of Yap1 (oxII-T) was equivalent to that of Yap1C¹⁷¹T (oxII) (~256 mV) (21), we expected that wild type Yap1 and Yap1C¹⁷¹T might show similar activities in ybp1-1 cells. However, transcriptional activation of the target gene and the resistance to H₂O₂ of Yap1C¹⁷¹T in ybp1-1 cells were lower than in wild type Yap1 in ybp1-1 cells (data not shown). Therefore, the six Cys residues of Yap1 might also be required for Tsa1-induced activation of Yap1 in ybp1-1 cells.

**DISCUSSION**

A.

**B.**

**FIGURE 9.** Tsa1-catalyzed formation of disulfide bonds in Yap1 results in a low level of the active form due to insufficient formation of reduction-resistant Yap1 (oxII). A, possible mechanism for Tsa1-induced disulfide bond formation (see "Discussion"); B, correlation between the levels of active forms of Yap1 and TRX2 transcriptional activation (y axis) and the time after the addition of H₂O₂ (x axis) for each disulfide bond formed in Yap1. The process and structure of Gpx3-induced oxidation of Yap1, which is indicated by pink arrows, were adapted from our previous report (21). The Tsa1-induced oxidation process in ybp1-1 cells is indicated by blue dotted lines. In this case, the ratio of active Yap1 (oxI-T) to reduced inactive Yap1 is lower because of the higher reduction potential of this form, which is probably determined by only one disulfide bond between the n-CRD and c-CRD. The increase in the level of Yap1 (oxI-T) may be dependent on the level of oxidized Trx. The possible ratio of Trx (ox) to Trx (red) is represented by the width of the blue block.

**FIGURE 9.** Tsa1-induced Disulfide Bond in Yap1 in ybp1-1 Cells
**Tsa4-induced Disulfide Bond in Yap1 in ybp1-1 Cells**

Atf4 system is also required at higher levels of \( \text{H}_2\text{O}_2 \). Therefore, in budding yeast, Yap1 may sense a broad range of intracellular levels of \( \text{H}_2\text{O}_2 \) by utilizing Gpx3, which is a hyperoxidation-resistant Prx (11).

The results presented here further support our model of redox shift-mediated sensing and transduction of peroxide stress (21), whereby we can explain how the activity of Yap1 is determined during the redox dynamic equilibrium controlled by the balance between Prx-dependent oxidation and Trx-dependent reduction.

**REFERENCES**

1. Halliwell, B., and Gutteridge, J. M. C. (1998) *Free Radicals in Biology and Medicine*, 3rd Ed., Oxford University Press, Oxford
2. Rhee, S. G. (2006) *Science* **312**, 1882–1883
3. Juarez, J. C., Manuia, M., Burnett, M. E., Betancourt, O., Boivin, B., Shaw, D. E., Tonks, N. K., Mazar, A. P., and Donate, F. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 7147–7152
4. Zhong, M., Aslund, F., and Storz, G. (1999) *Free Radic. Biol. Med.* **27**, 1718–1721
5. Aslund, F., Zhong, M., Beckwith, J., and Storz, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6161–6165
6. Kim, S. O., Merchant, K., Nudelman, R., Beyer, W. F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J. S. (2002) *Cell* **109**, 383–396
7. Tao, K. (1999) *FEBS Lett.* **457**, 90–92
8. Lee, C., Lee, S. M., Mukhopadhyay, P., Kim, S. J., Lee, S. C., Ahn, W. S., Yu, M. H., Storz, G., and Ryu, S. E. (2004) *Nat. Struct. Mol. Biol.* **11**, 1179–1185
9. Wood, Z. A., Schroder, E., Robin Harris, J., and Poole, L. B. (2003) *Trends Biochem. Sci.* **28**, 32–40
10. Wong, C. M., Siu, K. L., and Jin, D. Y. (2004) *J. Biol. Chem.* **279**, 23207–23213
11. Delaunay, A., Pfienger, D., Barrault, M. B., Ving, J., and Toledano, M. B. (2002) *Cell* **111**, 471–481
12. Tanaka, T., Izawa, S., and Inoue, Y. (2005) *J. Biol. Chem.* **280**, 42078–42087
13. Lee, S. Y., Song, J. Y., Kwon, E. S., and Roe, J. H. (2008) *Biochim. Biophys. Res. Commun.* **367**, 67–71
14. Bozonet, S. M., Findlay, V. J., Day, A. M., Cameron, J., Veal, E. A., and Morgan, B. A. (2005) *J. Biol. Chem.* **280**, 23319–23327
15. Vivancos, A. P., Castillo, E. A., Biteau, B., Nicot, C., Ayte, J., Toledano, M. B., and Hidalgo, E. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 8875–8880
16. Kuge, S., Toda, T., Iizuka, N., and Nomoto, A. (1998) *Genes Cells* **3**, 521–532
17. Yan, C., Lee, L. H., and Davis, L. I. (1998) *EMBO J.* **17**, 7416–7429
18. Wood, M. J., Andrade, C. E., and Storz, G. (2003) *Biochemistry* **42**, 11982–11991
19. Delaunay, A., Isnard, A. D., and Toledano, M. B. (2000) *EMBO J.* **19**, 5157–5166
20. Izawa, S., Maeda, K., Sugiyama, K., Mano, J., Inoue, Y., and Kimura, A. (1999) *J. Biol. Chem.* **274**, 28459–28465
21. Okazaki, S., Tachibana, T., Naganuma, A., Mano, N., and Kuge, S. (2007) *Mol. Cell* **27**, 675–688
22. Veal, E. A., Ross, S. J., Malakasi, P., Peacock, E., and Morgan, B. A. (2003) *J. Biol. Chem.* **278**, 30896–30904
23. Kuge, S., and Jones, N. (1994) *EMBO J.* **13**, 655–664
24. Kuge, S., Jones, N., and Nomoto, A. (1997) *EMBO J.* **16**, 1710–1720
25. Morgan, B. A., Banks, G. R., Toone, W. M., Raitt, D., Kuge, S., and Johnston, L. H. (1997) *EMBO J.* **16**, 1035–1044
26. Kuge, S., Arita, M., Murayama, A., Maeta, K., Izawa, S., Inoue, Y., and Nomoto, A. (2001) *Mol. Cell. Biol.* **21**, 6139–6150
27. Okazaki, S., Naganuma, A., and Kuge, S. (2005) *Antioxid. Redox Signal.* **7**, 327–334
28. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O’Shea, E. K., and Weissman, J. S. (2003) *Nature* **425**, 737–741
29. Dunn, B., and Wobbe, C. R. (1997) in *Current Protocols in Molecular Biology* (Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.1.1–13.1.3, John Wiley & Sons, Inc., New York
30. Azevedo, D., Tacnet, F., Delaunay, A., Rodrigues-Pousada, C., and Toledano, M. B. (2003) *Free Radic. Biol. Med.* **35**, 889–900
31. Wood, M. J., Storz, G., and Tjandra, N. (2004) *Nature* **430**, 917–921
32. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) *J. Biol. Chem.* **269**, 27670–27678
33. Jang, H. H., Lee, K. O., Chi, Y. H., Jung, B. G., Park, S. K., Park, J. H., Lee, J. R., Lee, S. S., Moon, J. C., Yun, J. W., Choi, Y. O., Kim, W. Y., Kang, J. S., Cheong, G. W., Yun, D. J., Rhee, S. G., Cho, M. J., and Lee, S. Y. (2004) *Cell* **117**, 625–635
34. Biteau, B., Labarre, J., and Toledano, M. B. (2003) *Nature* **425**, 980–984
35. Vivancos, A. P., Castillo, E. A., Jones, N., Ayte, J., and Hidalgo, E. (2004) *Mol. Microbiol.* **52**, 1427–1435
36. Veal, E. A., Findlay, V. J., Day, A. M., Bozonet, S. M., Evans, J. M., Quinn, J., and Morgan, B. A. (2004) *Mol. Cell* **15**, 129–139