Differential Oxidant Tolerance Determined by the Key Transcription Factor Yap1 Is Controlled by Levels of the Yap1-binding Protein, Ybp1*

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Background: Disulfide bond formation regulates nuclear localization of transcription factor Yap1.
Results: Levels of Yap1-binding protein Ybp1 control cytoplasmic oxidative protein folding.
Conclusion: Oxidative stress response limited by levels of protein folding cofactor rather than its transcriptional regulator target.
Significance: Oxidative protein folding is the key step in oxidative stress response in fungi.

The Saccharomyces cerevisiae transcription factor Yap1 is a central determinant of oxidative stress tolerance. This protein is found primarily in the cytoplasm in the absence of oxidative stress but, upon exposure to oxidants, rapidly translocates to the nucleus and activates expression of target genes. Although both diamide and H2O2 have been used to impose oxidative stress on nucleus and activates expression of target genes. Although both stress but, upon exposure to oxidants, rapidly translocates to the cytoplasm in the absence of oxidative stress, Yap1 is found primarily in the cytoplasm. In the absence of oxidative stress, Yap1 exists in a reduced form and these proteins form a stable complex.

Ybp1. In the current study, we have determined that two disulfide bonds linked cysteines 303 with 598 as well as Cys-310 with Cys-598. These disulfide bonds form between two cysteines present in the C-terminal cysteine-rich domain (C-CRD) of Yap1. The oxidized form of Yap1 that is catalyzed by the presence of Gpx3 and Ybp1. In the current study, we have determined that two distinct pools of Yap1 exist in the cell. These pools are designated by the level of Yap1. Yap1 interacts directly with Yap1 and these proteins form a stable complex in vivo. Genetic and biochemical experiments indicate that Yap1 is rate-limiting for Yap1 oxidative folding during H2O2 stress. The fungal pathogen Candida glabrata expresses a protein homologous to Yap1 called CgYbp1. Overproduction of CgYbp1 elevated H2O2 tolerance in this pathogen indicating that the determinant role of Yap1 in setting the level of H2O2 resistance has been evolutionarily conserved.

Oxidative stress is experienced by all cells growing in an aerobic environment. The inevitability of this environmental challenge has led to the elaboration of a wide range of strategies to deal with the production of potentially damaging reactive oxygen species (ROS). A central theme among the responses to ROS is the transcriptional reprogramming of cells to express new proteins that can act to detoxify these compounds. Control of the regulated response to elevated ROS levels is an important feature of all cells but especially in microorganisms because a key feature of the innate immune response in animals involves the generation of a bolus of ROS to eliminate these invading microbes (reviewed in Refs. 1 and 2).

The yeast Saccharomyces cerevisiae has served as a model eukaryotic cell for many fundamental processes and oxidative stress is no exception (3, 4). The transcription factor Yap1 is a central component in the oxidative stress response in this yeast (5, 6). Loss of YAP1 elicits an oxidant-hypersensitive phenotype and prevents ROS-induced gene expression. Regulation of Yap1 occurs in large part through control of the nuclear localization of this protein. In the absence of oxidative stress, Yap1 enters the nucleus at a relatively low basal rate compared with its rapid nuclear export via the exportin protein Crm1 (7, 8).

Treatments with oxidants (like diamide or diethylmaleate) that act to directly modify cysteine residues present in the C-terminal cysteine-rich domain (C-CRD) of Yap1 blocks the ability of the protein to associate with Crm1. In turn, the oxidized form of Yap1 accumulates in the nucleus and activates expression of particular target genes (9).

Interestingly, the response of Yap1 to oxidative stress initiated by H2O2 exposure is more complex. H2O2 challenge of cells causes a covalent intermediate to be formed between the glutathione peroxidase Gpx3 and a cysteine (Cys-598) in the C-CRD (10) in a manner dependent on the presence of the Yap1-binding protein Ybp1 (11, 12). Next, disulfide bonds are formed between two cysteines present in the C-CRD with two cysteines present in the N-terminal cysteine-rich domain with the concomitant release of Gpx3 (10, 13, 14). These disulfide bonds link cysteines 303 with 598 as well as Cys-310 with Cys-629. This uniquely folded form of Yap1 is also unable to interact with Crm1 and critically can recruit the transcriptional Medi-
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TABLE 1

| Strain | Description | Source |
|--------|-------------|--------|
| SEY6210 | MATα leu2–3, 112 ura3–52 his3Δ200 trplΔ901 lys2–801 suc2–Δ9 Mel+ | Scott Emr |
| SML13 | MATα leu2–3.112 ura3–52 his3Δ200 trplΔ901 lys2–801 suc2–Δ9 Mel+ yap1-1Δ::hisG | 20 |
| BY4742 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Open Biosystems |
| BY4742 ybp1Δ | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Open Biosystems |
| BY4742 YBP1-TAP | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBP1-TAP-HIS3MX6 | This study |
| BY4742 YBP1-TAP | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBP1-TAP-HIS3MX6 | This study |
| SLS1 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study |
| SLS3 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study |
| Cg 40F1 | Cgura Δ(–85 + 932):::Tn903Neo8 | David Soll |
| SL55 | Cgura Δ(–85 + 932)::Tn903Neo8 ybp1Δ::hapMX4 | This study |
| KG90 | 40F1 CgYBP1–1X HA URA3 | This study |
| KG91 | 40F1 CgPGK1-YBP1–1X HA URA3 | This study |

TABLE 2

| Plasmid | Description | Source |
|---------|-------------|--------|
| pNT13 | pRS316 GFP-YAP1 | 19 |
| pSC99 | pSEY102 TRX2-lacZ | 19 |
| YEp352-YAP1 | YEp352-YAP1 | Lab Plasmid |
| pJÅW139 | pET28h(+):: YAPI (63–650 amino acids) | Lab Plasmid |
| pKGE2 | pRS315 PGK1-1HA-YBP2 | 11 |
| pKGE3 | pRS315 PGK1-HA-YBP2 | 11 |
| pKGE4 | pRS315 PGK1-HA-GPX3 | 11 |
| pKGE50 | GFP-ΔNLS-YAP1 in pRS316 | This study |
| pKGE51 | pRS426 URA3 Cg YBP1–1XHA | This study |
| pKGE52 | pRS426 CgPGK-YBP1–1XHA | This study |
| pSL5 | pGEX6-P1-GST-CYBP1 | This study |
| pKGE60 | pET28h(+)–GPX3 | This study |
| pKGE61 | pET28h(+)–C629A YAPI | This study |

EXPERIMENTAL PROCEDURES

Yeast Strains and Methods—Strains used in this study are listed in Table 1. Yeast cells were grown either in rich medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose) for non-se-

ated component Rox3 to target promoters important in the response to H2O2-induced oxidative stress (15). These observations illustrate the additional components and added complexity of the response to H2O2 stress.

The role of Ybp1 in the H2O2-induced folding of Yap1 is not well understood. Ybp1 is not required for diamide resistance but is crucial for H2O2 tolerance (11, 12). We found that overproduction of Ybp1 but notably not Yap1 strongly elevated H2O2 resistance indicating that Ybp1 represented a limiting component in H2O2 resistance. Strikingly, overproduction of Ybp1 caused a diamide-hypersensitive phenotype compared with wild-type cells. Elevated dosage of Ybp1 also increased association of Ybp1 and Yap1 in vivo as well as accelerating H2O2-induced oxidative folding of Yap1 and target gene induction. Biochemical experiments indicated that Yap1 and Ybp1 directly associate in vitro. These data support the view that Yap1 exists in two different pools defined by association with Ybp1. The Yap1-Ybp1 complex is involved in the response to H2O2 but not diamide although non-Ybp1-associated Yap1 mediates diamide resistance but is less important in H2O2 tolerance. The levels of Ybp1 control the distribution of Yap1 between these two pools and the response to oxidative stress.

amplified and cloned in the YEp352 vector. The Ntl1 fragment containing the hygromycin cassette from the pAG32 plasmid (16) was inserted into this plasmid in place of the CgYBP1 coding sequences, yielding pSL66. The Kpn1-SphI fragment of pSL66 plasmid was then transformed in C. glabrata 40F1 strain and screened for hygromycin resistance. To construct a C. glabrata 40F1 strain carrying a C-terminal-tagged CgYBP1-1X HA allele under control of either the native or the strong CgPGK1 promoter, the plasmid pKGE51 was cut within the CgYBP1 ORF by enzymes Clal and BstELI, whereas plasmid pKGE52 was cut within the CgPGK1 promoter by AvrII. The linearized plasmids were then transformed in the C. glabrata 40F1 strain to generate KG90 (CgYBP1–1X HA URA3) and KG91 (CgPGK1-CgYBP1–1X HA URA3). C. cerevisiae transformation was carried out by the lithium acetate technique of Ito et al. (17) and C. glabrata transformation was performed as described by Ueno et al. (18).

Plasmids—Plasmids used in this study are listed in Table 2. To express a GST-Ybp1 fusion protein, a BamHI-Sall fragment corresponding to ScYBP1 or CgYBP1 ORF was ligated into the BamHI and SalI sites of pGEX-6P1 (Amersham Biosciences). To construct a mutant Yap1 lacking a NLS, a PCR fragment carrying a truncation from residues 7 to 61 in the Yap1 N terminus was amplified using primers Yap1-NLS-For (CCAGAT- CATATGAAAACAACTGACTTTTTCCAAGTCTGCC) and Yap1-NLS-Rev (GGGCCGCCATTTGGCCAGTCTCTT- CTGCTTAGTTCCAGGATCACAATTCATCAGCCATCCATTGG). This PCR fragment was then recombined in plasmid pNT13 (19) to generate a mutant GFP-Yap1 derivative lacking the nuclear localization signal. To generate plasmids carrying C-terminal 1X HA-tagged alleles of the C. glabrata YBP1 ORF, primers were used to amplify CgYBP1 ORF from genomic DNA isolated from wild-type C. glabrata

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strain 40F1. Cg-PGK YBP1 F1 and Cg-PGK YBP1 R1 to amplify 2-kb ORF without promoter or using primers CgYBP1-promoter-F1 and Cg-PGK YBP1 R1 to amplify a 2.5-kb cassette containing both the YBP1 ORF along and its native promoter. These PCR cassettes were then recombined in plasmid pLS20 (Cg PGK-PSD1–1XHA) excised with BamHI-Sall to yield pRS426 Cg YBP1-XHA and pRS426 Cg PGK-YBP1-1X HA plasmids. A plasmid expressing Gpx3 in bacteria was constructed by inserting the Gpx3 ORF as a BamHI fragment into pET28b (+) at the BamHI site to generate pKGE60. Expression of C629A Yap1 in bacteria was accomplished by use of a pET28b (+)-C629AYap1 construct. This plasmid was generated by ligating a KpnI-NotI fragment isolated from plasmid pRS316-C629A YAP1 (20) into the plAW139 plasmid excised with the same enzymes, generating pKGE61.

Western Blot Analysis—Cells grown in 10 ml to mid-log phase were harvested, washed, and lysed by either glass bead lysis in the presence of TCA or in buffer containing 300 mM sorbitol, 100 mM NaCl, 5 mM MgCl2, 10 mM Tris (pH 7.4), and protease inhibitors. Cell lysates were cleared and the Bradford protein assay (Bio-Rad) was used to determine the protein concentration. Equal portions of protein from each sample were run on a SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane, blocked with 5% nonfat dry milk in phosphate-buffered saline, and probed with polyclonal anti-Yap1 antibody (20), polyclonal anti-TAP antibody (Open Biosystems), or monoclonal anti-HA antibodies (Covance). Horseradish peroxidase-conjugated secondary antibody and the ECL kit (Pierce) were used to visualize immunoreactive protein.

β-Galactosidase Assays—Wild-type cells were transformed with low-copy number plasmids corresponding to the empty pRS315 vector or pRS315-PGK1-YBP1 plasmid along with the TRX2-lacZ gene fusion construct. Transformants were grown to mid-log phase in selective media and then either left untreated or induced by adding oxidants at the indicated concentrations for an additional 1 h. An equal number of cells from each sample was harvested at various time points, washed, and assayed for β-galactosidase activity as described (21). All assays were carried out in triplicate and represented at least three independent trials.

Co-immunoprecipitation—All immunoprecipitation assays were performed using lysed spheroplasts. To obtain spheroplasts, 300 ml of cells were harvested at A600nm ~ 0.8 and washed with 10 ml of solution 1 (1 M sorbitol, 10 mM MgCl2, 30 mM DTT, 100 μg/ml of phenylmethylsulfonyl fluoride, 50 mM K2HPO4) and followed by centrifugation. The pellet was resuspended in 10 ml of solution 2 (1 M sorbitol, 10 mM MgCl2, 30 mM DTT, 100 μg/ml of phenylmethylsulfonyl fluoride, 50 mM K2HPO4, 25 mM sodium succinate, pH 5.5) containing oxaMI-case and incubated at 30 °C for 1 h. The resulting spheroplasts were pelleted by centrifugation at 5,000 × g for 20 min at 4 °C. Protein lysates were prepared by lysing these spheroplasts with lysis buffer (1% Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.2) containing 1X protease inhibitor mixture, followed by addition of 2 mM EDTA, 200 mM sodium vanadate, 50 mM sodium fluoride, and 1 mM DTT. Lysis was performed by shaking cell suspensions on a Tomy shaker in the presence of glass beads for 5 min at 4 °C. Protein extracts were clarified by centrifuging at 14,000 × g for 5 min at 4 °C. For immunoprecipitation, washed protein A beads were incubated with cell lysates for 2 h at 4 °C, then anti-TAP antibody was added to the lysates and incubated at 4 °C overnight. This mixture was centrifuged at 14,000 × g for 5 min at 4 °C, and 50 μl of supernatant was saved as a control. After washing the beads three times with PBS (pH 7.0) containing 1 mM DTT, immunoprecipitated proteins were recovered by adding 3X Laemmli dye (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol, 2% bromphenol blue dye). These precipitates along with the inputs and other control proteins were then loaded on an 8% polyacrylamide gel and analyzed by Western blotting using a polyclonal anti-Yap1 antibody.

GST Interaction Assays—To prepare protein extracts, yeast cells were grown to mid-log phase, and 10 mM sodium azide and 10 mM potassium fluoride were directly added to the culture. After chilling on ice for 5 min, cells were harvested and resuspended with 4 M sorbitol buffer containing 1X protease inhibitor mixture (Roche Applied Science) and 2 mM DTT. After lysing the cells on a Tomy shaker in the presence of glass beads at 4 °C for 45 min, the extract was clarified by centrifugation. The resulting cell extract was pre-cleared by rotation with the washed glutathione-Sepharose 4B beads for 1 h at 4 °C, followed by centrifugation. To prepare crude extracts from cells expressing recombinant Yap1 proteins, a pET28b+ plasmid expressing Yap1 amino acid residues 63–650 (pJAW139) or a similar clone expressing the C629A form of Yap1 were transformed into BL21/DE3 bacterial cells. These transformants were induced with isopropyl 1-thio-β-d-galactopyranoside (Research Products International) and protein extracts were prepared. To decrease nonspecific interaction, the crude extract was pre-cleared by incubating for 1 h with glutathione beads, onto which recombinant GST had been bound, followed by centrifugation. GST fusion proteins were expressed and purified from bacterial lysates as described (22). The GST fusion proteins were incubated with washed glutathione-Sepharose 4B beads for 1 h. These beads were further incubated with the pre-cleared extracts for 3 h at 4 °C and then spun down at 13,000 × g for 5 min and washed three times with C buffer (25 mM HEPES, pH 7.6, 10% glycerol, 50 mM KCl, 0.1 mM EDTA) containing 2 mM DTT. The bound proteins were eluted by 50 μl of PBS, 50 mM reduced glutathione (pH 7.2). Samples were then loaded on 10% polyacrylamide gel and analyzed by Western blotting using anti-TAP (1:1000, Open Biosystems) or anti-polycyonal Yap1 antibodies (1:1500).

The experiments using Gpx3 were carried out by loading the GSH-Sepharose beads with GST-Ybp1 and Yap1 as above. Extracts from either pET28b (+) or pKGE60 (Gpx3)-containing cells were prepared as described above. Protein extracts were split into equal fractions and then incubated at room temperature for 10 min in the presence or absence of oxidants (1 mM of either diamide or H2O2). These extracts were then passed over the Yap1-containing beads, eluates were collected and precipitated with TCA. In some cases, the proteins remaining after treatment with the extracts were eluted by addition of reduced GSH, followed by TCA precipitation. Precipitates were

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washed with acetone and analyzed by Western blotting using anti-Yap1 and anti-GST antisera.

Analysis of Yap1 Oxidation Status—To monitor status of Yap1 oxidation, we followed the method described earlier (13). After exposure to 1 mM H$_2$O$_2$ for the indicated time periods, 50 ml of cells were collected and washed with 20% TCA. The pellets were then resuspended in 12.5% TCA, glass beads were added and vortexed vigorously in a Tomy shaker for 10 min. After breakage, the extracts were collected and beads were washed again with 12.5% TCA. Beads and debris were removed by centrifugation, the supernatant was collected and pooled with the initial extract. Proteins were pelleted by high speed centrifugation and washed with ice-cold acetone twice, followed by air drying for 20 min. Dried pellets were then dissolved in 75 mM iodoacetamide, 1% SDS, 100 mM Tris-HCl (pH 8), 1 mM EDTA, complete protease inhibitors (Roche Applied Science), and incubated at 25 °C for 15 min. Samples were then subjected to dialysis against 10 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM MgCl$_2$ for 4 h. Protein extracts were boiled in 3× Laemmli buffer (without β-mercaptoethanol) and separated by nonreducing SDS-PAGE. The separated proteins were then transferred to nitrocellulose membrane and immunoblotted using anti-Yap1 serum.

Fluorescence Microscopy—To examine the localization of Yap1–GFP fusion proteins, cells were grown to an A$_{600}$ of ~0.6 and first stained with 4′,6′-diamidino-2-phenylindole (DAPI) for 1 h to visualize the nuclei and mitochondria. Cells were then exposed to hydrogen peroxide or diamide for 20 min. Equal amounts of cells were spun down, washed, and resuspended in 50 μl of water. 8 μl of concentrated culture were visualized using an Olympus BX60 microscope. Images were captured on a Hamamatsu digital camera.

RESULTS

Ybp1 Is Limiting for H$_2$O$_2$ Resistance in S. cerevisiae—Given that several factors are required for Yap1-mediated H$_2$O$_2$ tolerance, we wanted to determine which of these might be limiting for this oxidative stress phenotype. Wild-type cells were transformed with plasmids that placed the various components involved in the Yap1-mediated response to H$_2$O$_2$ under control of the strong constitutive PGK1 promoter. Transformants were then tested for the ability to grow on medium containing H$_2$O$_2$ (Fig. 1A).

Transformants expressing Ybp1 under control of the strong PGK1 promoter exhibited robust resistance to H$_2$O$_2$. Similar transformants producing driving Gpx3 or the Ybp1 homologue Ybh1 from the PGK1 driving Ybp1, Ybh1 (YBP1 homologue involved in centromere function (11, 12, 32)), or GPX3 were introduced into wild-type (SEY6210) S. cerevisiae cells. Additionally, a high-copy number vector plasmid (Yep352) or this same plasmid containing the YAP1 gene were similarly introduced into SEY6210 cells. Transformants were grown to mid-log phase, placed on medium containing H$_2$O$_2$, and photographed after incubation at 30 °C. B, whole cell protein extracts were prepared from transformants described above containing the indicated expression plasmids. Equal aliquots of extracts were separated on SDS-PAGE and analyzed by Western blotting with an anti-HA antibody. Each expression plasmid contains two HA epitopes fused to the N terminus of the PGK1-driven protein. Molecular mass of the recombinant proteins is indicated in kDa on the left side of the panel. Ponceau S staining of total protein is shown at the bottom of panels B and C to confirm equal loading. C, either wild-type (WT:SEY6210) or isogenic yap1Δ cells transformed with the indicated high-copy number plasmids were grown to mid-log phase, protein extracts were prepared and analyzed by Western blotting using anti-Yap1 antibody (20).

Because Ybp1 overproduction led a dramatic increase in H$_2$O$_2$ tolerance, these same cells were evaluated for their resistance to the oxidant diamide. Our expectation was that Ybp1 overproduction would have negligible influence on diamide resistance because loss of the YBP1 gene has been shown to specifically depress H$_2$O$_2$ but not diamide tolerance (11, 12). Wild-type cells transformed with the low-copy number vector plasmid or this same plasmid containing the PGK1-YBP1 fusion gene were grown to mid-log phase and then placed on medium containing H$_2$O$_2$, diamide, or lacking any oxidant. These transformants were allowed to grow and then photographed (Fig. 2A).
Surprisingly, we found that Ybp1 overproduction caused a reduction in diamide resistance while still increasing H$_2$O$_2$ tolerance. These data indicate that high levels of Ybp1 cause an increase in H$_2$O$_2$ resistance with a concomitant decrease in diamide tolerance. To ensure that the observed increase in H$_2$O$_2$ exhibited the expected Yap1 dependence, the PGK1-YBP1 plasmid was introduced into a yap1/H9004 strain along with several control plasmids. Transformants were tested for the ability to grow in the presence of H$_2$O$_2$ (Fig. 2B). The absence of Yap1 completely blocked the ability of the PGK1-YBP1 fusion gene to elevate H$_2$O$_2$ resistance above transformants containing the pRS315 vector alone. Only returning the YAPI gene to this strain was sufficient to elevate H$_2$O$_2$ resistance. The ability of Ybp1 overproduction to increase H$_2$O$_2$ resistance was strictly dependent on the presence of Yap1.

Expression of Yap1 has also been demonstrated to markedly elevate drug resistance (23–25). Overproduction of Yap1 elevated cycloheximide resistance in wild-type cells (Fig. 2C). However, elevated expression of Ybp1 lowered the ability of cells to tolerate cycloheximide as seen above for resistance to the oxidant diamide. These data argue that the interaction of Ybp1 and Yap1 has similar negative effects on diamide and drug resistance but very different consequences to H$_2$O$_2$ tolerance.

Elevated Levels of Ybp1 Differentially Influence Yap1-mediated Response to Oxidants—Previous studies (19, 26) have established that Yap1 activation of transcription of the thioredoxin-encoding TRX2 gene is critical for H$_2$O$_2$ resistance. To determine whether overproduction of Ybp1 influenced TRX2 inducible expression, a low-copy number plasmid carrying a TRX2-lacZ reporter gene along with a vector plasmid (pRS315) or the same vector containing a fusion gene overproducing Ybp1 (PGK1-YBP1). Transformants were grown to early log phase and challenged with H$_2$O$_2$ or left untreated. At the indicated time points, samples were withdrawn and TRX2-dependent β-galactosidase activity was measured. The same protocol as in A was followed except oxidative stress was imposed with the addition of diamide.

**FIGURE 2. Overproduction of Ybp1 inhibits diamide and cycloheximide resistance.** A, wild-type cells were transformed with the indicated plasmids. Transformants were tested as above for the ability to tolerate H$_2$O$_2$ or diamide. B, a strain lacking Yap1 (yap1Δ) was transformed with the indicated plasmids. Transformants were placed on rich medium containing H$_2$O$_2$ to evaluate their relative resistance to this oxidant. C, isogenic wild-type cells or cells overexpressing Ybp1 (TDH3-YBP1) were transformed with an empty high-copy number vector plasmid (YEp351) or the same plasmid containing the wild-type YAP1 gene (YEp351-YAP1). Transformants were grown to mid-log phase and then placed on rich medium containing a gradient of cycloheximide (Cyh).

**FIGURE 3. Elevated Ybp1 levels exhibit differential effects on inducible gene expression.** A, wild-type cells were transformed with a low-copy number plasmid carrying a TRX2-lacZ reporter gene along with a vector plasmid (pRS315) or the same vector containing a fusion gene overproducing Ybp1 (PGK1-YBP1). Transformants were grown to early log phase and challenged with H$_2$O$_2$ or left untreated. At the indicated time points, samples were withdrawn and TRX2-dependent β-galactosidase activity was measured. B, the same protocol as in A was followed except oxidative stress was imposed with the addition of diamide.
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After 15 and 30 min of diamide exposure, these transformants were grown to early log phase and then treated with diamide. plasmid into a wild-type strain. These double transformants exhibited 55 units/H18528 after 45 min of diamide treatment, whereas wild-type cells only exhibited 90 units/A660, whereas wild-type cells only exhibited 55 units/A660.

C629A Yap1 was unable to respond to H2O2 challenge although this mutant protein caused cells to be hyper-resistant to diamide. The failure of C629A Yap1 to confer H2O2 resistance could be due to this mutant form being unable to interact with Yap1 or simply being restricted to a different compartment of the cell than Yap1. Use of this cell-free interaction assay allowed discrimination between these two different hypotheses underlying the H2O2 defect of C629A Yap1.

The GST-Ybp1 fusion protein or GST alone was bound to GSH-Sepharose beads. Bacterially expressed polyhistidine-Yap1 or C629A Yap1 was then passed over these beads. Unbound proteins were collected and the beads were washed extensively with buffer. Specifically bound proteins were eluted by treating the beads with reduced GSH, collected, and subjected to Western blotting using the anti-Yap1 antibody (Fig. 6A).

Beads with GST-Ybp1 bound efficiently captured recombinant Yap1 that could be eluted with GSH. Importantly, recombinant C629A Yap1 was able to directly associate with Yap1 supporting the view that this mutant protein could interact with Yap1 in vivo but their different subcellular distributions precludes this. Conversely, beads with only GST bound failed to interact with bacterially produced Yap1. This result indicates that Yap1 and Ybp1 can directly interact and no additional yeast factor is required for this complex to form.

To test if increasing the dosage of Ybp1 would lead to enhanced Yap1-Ybp1 complex formation, we constructed a
strain containing a YBP1-TAP fusion gene in place of the wild-type YBP1 locus. The YBP1 promoter was then replaced with the TDH3 promoter to overproduce the Ybp1-TAP fusion protein. Additionally, we constructed a yap1Δ derivative in the presence of the YBP1-TAP fusion gene. These three strains were grown to mid-log phase, whole cell protein extracts were prepared, and the Ybp1-TAP protein was recovered by immunoprecipitation using the anti-TAP antibody. These immunoprecipitates were separated on SDS-PAGE and subjected to Western blotting analysis using either the anti-Yap1 or anti-TAP antibodies (Fig. 6B).

Elevating the expression of Ybp1-TAP led to increased Yap1 association with Ybp1. Steady-state levels of Yap1 were unaffected by the overproduction of Ybp1-TAP. These data provide further support for the idea that the level of the Yap1-Ybp1 complex represents the limiting parameter for H2O2 tolerance. Increasing the level of this complex prior to H2O2 exposure leads to an increased ability to tolerate this oxidant but decreases resistance to diamide.

**Dissociation of the Yap1-Ybp1 Complex in Vitro**—Our finding that Yap1 and Ybp1 can directly interact in vitro provided the possibility to examine the regulation of this binding interaction. Previous work provided important insight into the oxidative folding of Yap1. The glutathione peroxidase homologue Gpx3 is covalently linked to Yap1 in the presence of H2O2 and Ybp1 (10). Structural analysis indicated that the H2O2-oxidized form of Yap1 is likely to undergo dramatic changes in the tertiary structure of the factor because of the formation of two new disulfide bonds between the N- and C-CRD regions (14). This likely structural reorganization suggested the possibility that the Yap1-Ybp1 complex might dissociate upon H2O2 oxidation.

To examine this possibility, we formed the GST-Ybp1-Yap1 complex on Sepharose-GSH beads as described above. These

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**FIGURE 5. Oxidative folding of Yap1 responds to Ybp1 dosage.** Increased Ybp1 expression inhibits diamide-induced nuclear translocation of Yap1. Wild-type cells were transformed with a low-copy number plasmid expressing GFP-Yap1 and either a vector plasmid (pRS315) or pRS315 carrying the PGK1-YBP1 fusion gene. Transformants were grown to early log phase and then subjected to diamide stress for the times indicated on the left. Cells were withdrawn and visualized for GFP-Yap1 (GFP), DNA (DAPI), or by Nomarski optics (DIC). Quantitation of the fluorescence pattern seen in 200 representative cells is presented at the bottom of the figure.
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FIGURE 6. Yap1 and Ybp1 interact in vitro and in vivo. A, recombinant forms of Yap1 (pET-YAP1) and Ybp1 (GST-Ybp1) were prepared in Escherichia coli and purified by standard techniques. Glutathione S-transferase (GST) or GST-Ybp1 were bound to glutathione-Sepharose beads. Next, protein lysates from cells containing an empty expression vector (pET28a+) or the same vector driving bacterial expression of Yap1 (pET-YAP1) or a constitutively nuclear mutant form (C629A YAP1) were applied. An aliquot of the lysate (input) was blotted to estimate binding efficiency. After binding, unbound material was collected (FT) and the beads were washed with buffer (wash). Finally, bound material was eluted with reduced glutathione (Eluted). Samples of each fraction were resolved on SDS-PAGE and analyzed by Western blotting with the anti-Yap1 antibody. B, isogenic wild-type or yap1Δ cells containing the indicated YBP1-TAP fusion genes were grown to mid-log phase and protein extracts were prepared. Equal aliquots of these extracts (input) were retained to permit confirmation of the presence of each protein. Ybp1-TAP was immunoprecipitated using the anti-TAP antibody. These immunoprecipitates (IP), together with the input samples, were resolved on SDS-PAGE and analyzed by Western blotting (IB) using the indicated antibodies.

beads represent the starting material for the subsequent assays. Whole cell protein extracts were prepared from bacterial cells containing an empty expression vector (pET28b(+)) or the same plasmid expressing S. cerevisiae Gpx3 (pET-Gpx3). These protein extracts were treated with 1 mM H2O2 or used directly with no additions. Elution of Yap1 was accomplished by the addition of appropriately prepared protein extracts to the beads. The eluate was collected from each column and concentrated by TCA precipitation. The precipitates were then subjected to Western blotting using the anti-Yap1 antibody (Fig. 7A).

Addition of bacterial extracts containing Gpx3 induced a small but reproducible amount of elution of Yap1 from the beads. Importantly, incubation of these same extracts in the presence of 1 mM H2O2 released ~10 times as much Yap1, consistent with a requirement for this oxidant in the reaction. Use of the same amount of extract from cells containing the empty expression vector led to release of very small amounts of Yap1, either in the presence or absence of H2O2 treatment.

To further examine the specificity of this in vitro reaction, the ability of diamide to influence Ybp1-Yap1 association was compared with H2O2. Reactions were performed as described above except that 1 mM diamide was used to treat extracts to determine whether any oxidant could trigger Yap1 release from this column. As can be seen in Fig. 7B, exposure of the Gpx3-containing extract to diamide failed to induce any significant Yap1 release. To confirm that equal levels of Yap1 were present in all reactions, the remaining Yap1 was eluted by treatment with reduced GSH. To ensure that release of Yap1 from the column was not accompanied by simultaneous release of GST-Ybp1, a polyclonal anti-GST antibody was used to probe the samples in Fig. 7B. No significant release of the GST fusion protein was detected upon treatment with Gpx3 and H2O2 (data not presented).
shown), consistent with the interpretation that the effect of Gpx3 and H2O2 acts on Yap1 as expected. These data support the view that the presence of Gpx3 and H2O2 triggers a change in Yap1 that in turn leads to dissociation of the Ybp1-Yap1 complex, likely by triggering the oxidative folding of Yap1. Further experiments are underway to confirm this suggestion.

Yap1 Is Folded by Ybp1 in the Cytoplasm—Although Ybp1 is clearly required for H2O2-induced folding of Yap1 in the cell, the compartment in which this folding takes place is unknown. To determine whether Ybp1 could fold Yap1 in the cytoplasm, a mutant form of Yap1 was constructed that lacked the previously described nuclear localization signal (NLS) of this transcription factor (27). The intent was to trap Yap1 in the cytoplasm, then challenge cells with H2O2 and assess the ability of Ybp1 to fold cytoplasmically localized Yap1. Yap1 contains a bipartite NLS in its amino terminus between residues 5 and 59. This region of the protein was removed in the context of a GFP-Yap1 fusion protein to allow visualization of the localization defect in the ΔNLS mutant protein. The GFP-ΔNLS-Yap1 protein was expressed from the native YAP1 promoter and carried on low-copy number plasmid. This mutant Yap1 allele, along with the wild-type locus, was transformed into a yap1Δ strain. Transformants were grown to mid-log phase, and then allowed to grow at 37 °C (Fig. 9A). The defect caused by loss of the Yap1 NLS reduced complementation of the H2O2-sensitive phenotype by this plasmid-borne allele to a level indistinguishable from a strain lacking any functional Yap1. Consistent with the loss of complementation, the GFP-ΔNLS-Yap1 also failed to detectably accumulate in the nucleus upon treatment with H2O2 (Fig. 8B). The wild-type GFP-Yap1-expressing plasmid was able to carry out both of these functions. These results support the view that the GFP-ΔNLS-Yap1 is unlikely to enter the nucleus at a significant rate and exists primarily if not exclusively in the cytoplasm.

Having established the severity of the nuclear localization defect of GFP-ΔNLS-Yap1, the ability of this mutant form of Yap1 to be oxidatively folded was assessed. These transformants were treated with H2O2 and protein extracts were prepared under nonreducing conditions as described above. Levels of oxidatively folded Yap1 were determined by Western blotting after electrophoresis through a nonreducing SDS-PAGE (Fig. 8C).

H2O2-induced folding of the GFP-ΔNLS-Yap1 was not disturbed when compared with wild-type Yap1. This result argues that Ybp1-catalyzed folding of Yap1 can occur in the cytoplasm prior to nuclear localization of the protein during oxidative stress.

Conservation of the Role of Ybp1 in C. glabrata—The pathogenic yeast C. glabrata expresses a protein closely related to S. cerevisiae Yap1 called CgYap1 (28). Similarly, the C. glabrata genome contains a protein exhibiting striking sequence identity with Ybp1 encoded by a gene called CAGL0K06743. We will refer to the protein product of this gene as CgYbp1. To determine whether CgYbp1 was playing a similar role in H2O2 resistance in C. glabrata, several strains of this yeast were constructed. First, a null allele lacking the entire coding sequence of CgYBP1 was generated by replacing the ORF with a hygromycin resistance cassette. To enable detection of CgYbp1 expression, a HA tag was introduced at the C terminus of the chromosomal CgYBP1 gene. Finally, this CgPGK1 promoter was inserted into the CgYbp1-HA strain in place of the unmodified wild-type CgYbp1 promoter. These strains allowed the dosage of CgYbp1 to be varied across a wide range of expression values.

This set of strains, along with the unmodified wild-type strain, were grown to mid-log phase, placed on medium containing either H2O2 or diamide, and then allowed to grow at 37 °C (Fig. 9A). Loss of CgYbp1 from the cell resulted in a striking defect in H2O2 tolerance but had no significant influence on diamide resistance. Similar to the case in S. cerevisiae, replacing...
The data indicate that the action of Ybp1 is conserved in S. cerevisiae and the pathogen, C. glabrata.

**DISCUSSION**

Our finding that Yap1 exists in two distinct pools adds a level of fine control to the redox response controlled by this transcription factor. We divide these pools into the cycling or Ybp1-bound pool along with the Yap1-free pool. The cycling pool represents Yap1 that moves from the cytoplasm to the nucleus and back. This pool is responsible for diamide resistance and represents the major fraction of Yap1 in the cell. The Ybp1-bound pool of Yap1 is present as a stable subpopulation and corresponds to the minor fraction of Yap1. The Ybp1-bound pool mediates the response to H$_2$O$_2$ challenge. These two pools can be selectively activated in an oxidant-dependent fashion to induce genes relevant to detoxification of a given oxidative stress.

Our data also suggests very different behaviors of these different pools in the presence of different oxidants. For example, although the Ybp1-bound pool represents the minority of Yap1, overexpression of Ybp1 leads to near complete conversion of Yap1 to the H$_2$O$_2$-folded form in the presence of this oxidant (Fig. 4). We suggest that the initial Ybp1-bound Yap1 is folded and then released from Ybp1. This free Yap1 can then recruit more Yap1 from the cycling pool to continue production of the H$_2$O$_2$-folded form. This ultimately will convert all Yap1 into the H$_2$O$_2$-folded form and is likely at the root of eliciting diamide hypersensitivity upon Ybp1 overproduction. Elevated levels of Ybp1 cause progressively more Yap1 to be associated with Ybp1. This in turn lowers the level of the cycling pool of Yap1, leading to a diminished ability to mount a transcriptional response to diamide stress.

Conversely, diamide challenge of cells causes nuclear recruitment of the cycling pool of Yap1 but is unlikely to extract Yap1 from the Ybp1-bound pool. This assertion is supported by the observation that overproduction of Ybp1 caused diamide hypersensitivity. This is most consistent with the notion that the Ybp1-Yap1 complex remains stable even during diamide challenge.

The data reported in Fig. 6 provide the first demonstration that Yap1 and Ybp1 are likely to directly interact in the cell. We and others have been able to document this interaction via the use of two hybrid approaches (11, 12). However, our efforts to further localize the interaction motifs of these two proteins were unsuccessful, although both GST pulldown and two hybrid strategies were employed (data not shown). Small modifications of the Ybp1 protein sequence eliminated any detectable interaction with Yap1. We suggest that interaction of Yap1 and Ybp1 is likely to involve multiple, low-affinity interactions of these two proteins. The large rearrangement that occurs in the Yap1 structure upon H$_2$O$_2$-induced folding (14) is likely to trigger release of the folded protein from its Ybp1 partner. This suggestion is fully consistent with the release of Yap1 from the Ybp1-Yap1 complex immobilized on Sepharose-GSH beads shown in Fig. 7 upon the addition of Gpx3 and H$_2$O$_2$. Evidence in support of this notion comes from the observation that, whereas Yap1 clearly accumulates in the nucleus upon H$_2$O$_2$ stress (13, 19), Ybp1 does not (11, 12). It is important to recognize that further work is required to confirm that our in vitro reaction faithfully recapitulates H$_2$O$_2$-induced Yap1 folding. Direct biochemical confirmation such as done before (29) will be required to validate that the correct disulfide bonds are being produced in our in vitro reaction system.

Previous analyses were not able to clearly define localization of Ybp1 in the cell (11, 12). Our finding that a form of Yap1 that is unable to be delivered to the nucleus can be folded by Ybp1 in the presence of H$_2$O$_2$ confirms the ability of this regulatory step to occur in the cytoplasm. When cells initially experience oxidative stress, one of the first compartments to acquire an oxidizing potential will be the cytoplasm. Positioning the Yap1-Ybp1 complex in this compartment would allow oxidant sensing to occur relatively early. This is crucial because Yap1 does not directly detoxify oxidants but instead must move to the nucleus and induce gene expression. Coupling this geographical positioning to the high H$_2$O$_2$ reactivity of the Gpx3 sensor protein (discussed in Ref. 3) helps trigger Yap1 induction early in the onset of oxidative stress.
Mutant forms of Yap1 that constitutively localize in the nucleus confer hyper-resistance to diamide but fail to normally complement the H$_2$O$_2$ hypersensitivity of a yap1Δ strain (13, 19, 20, 30). A striking feature of these mutants is the range of different changes that produce the same phenotypic behavior: constitutive nuclear localization, diamide hyper-resistance, and H$_2$O$_2$ hypersensitivity. These mutations include small and large deletions as well as a variety of point mutations in various residues. Some of these mutants retain the various cysteine residues known to be required for proper oxidative folding of Yap1. A simplifying model for the consistent behavior of this wide range of mutation types is suggested by all of these mutants lacking normal Crm1 association. This would explain their nuclear localization. Combined with our finding that Ybp1 can fold Yap1 in the cytoplasm and the ability of C629A Yap1 to interact with Ybp1, these data suggest that the defect in H$_2$O$_2$-triggered folding in these C-terminal Yap1 mutant derivatives might be caused by separation of the cytoplasmic folding machinery from the constitutively nuclear Yap1.

Although Yap1 is clearly a key player in the response to oxidative stress, it is important to note that early identification of the YAPI gene was driven by its role in drug resistance (23). Overproduction of Yap1 strongly elevated resistance to drugs such as cycloheximide and sulfometuron methyl, neither of which is believed to have a significant contribution from oxidative stress in terms of their toxicity. This is most consistent with an increase in the cycling pool of Yap1 leading to an elevation in expression of drug resistance genes in a fashion similar to the observed increase in resistance to thiol-reactive compounds like diethylmaleate or diamide. Evidence that this same cycling pool is also required for drug resistance is provided by the observation that increased Ybp1 levels also diminished cycloheximide resistance in a manner similar to that seen for diamide tolerance (Fig. 2C). This observation is also consistent with our suggestion that these two pools of Yap1, defined by Ybp1, carry out different roles in the cell.

Homologues of Ybp1 can be found in Candida albicans as well as in S. cerevisiae and C. glabrata. It is interesting to note that Schizosaccharomyces pombe has a Yap1 homologue called Pap1 but no recognizable Ybp1-like protein encoded by its genome. Regulation of Pap1 and Yap1 is very similar, but S. pombe surprisingly lacks this important modulator of H$_2$O$_2$-induced folding. Pap1 is translocated to the nucleus upon H$_2$O$_2$ stress and contains analogous cysteine residues to those of Yap1 (31). The different environments faced by S. cerevisiae and its Candida relatives compared with those encountered by S. pombe may have inspired these different solutions to the problem of oxidative folding of Yap1.

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