Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*

Orna Carmel-Harel,1 Robert Stearman,1 Audrey P. Gasch,2,† David Botstein,3 Patrick O. Brown2 and Gisela Storz1,*

1Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-5430, USA.
2Department of Biochemistry, Stanford University School of Medicine, Howard Hughes Medical Institute, Stanford, CA 94305-5428, USA.
3Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305-5120, USA.

Summary

The *Saccharomyces cerevisiae* Yap1p transcription factor is required for the H2O2-dependent activation of many antioxidant genes including the TRX2 gene encoding thioredoxin 2. To identify factors that regulate Yap1p activity, we carried out a genetic screen for mutants that show elevated expression of a TRX2–HIS3 fusion in the absence of H2O2. Two independent mutants isolated in this screen carried mutations in the TRR1 gene encoding thioredoxin reductase. Northern blot and whole-genome expression analysis revealed that the basal expression of most Yap1p targets and many other H2O2-inducible genes is elevated in Δtrr1 mutants in the absence of external stress. In Δtrr1 mutants treated with H2O2, the Yap1p targets, as well as genes comprising a general environmental stress response and genes encoding protein-folding chaperones, are hyperinduced. However, despite the elevated expression of genes encoding antioxidant enzymes, Δtrr1 mutants are extremely sensitive to H2O2. The results suggest that cells lacking thioredoxin reductase have diminished capacity to detoxify oxidants and/or to repair oxidative stress-induced damage and that the thioredoxin system is involved in the redox regulation of Yap1p transcriptional activity.

Introduction

Aerobically growing cells are continuously challenged by the formation of reactive oxygen species (ROS) that arise from an incomplete reduction of molecular oxygen during respiration. If not eliminated, ROS are dangerous to cells as they can damage all cellular components (reviewed by Halliwell and Gutteridge, 1999). Most organisms possess protective antioxidant molecules and enzymes, and many cells are able to adapt to oxidative stress by increasing the levels of antioxidant enzymes. For example, *Saccharomyces cerevisiae* cells treated with low doses of H2O2 adapt and become resistant to otherwise lethal doses of H2O2 by increasing the transcription of many antioxidant enzymes (reviewed by Jamieson, 1998).

Yap1p (yeast AP-1) is critical in regulating the *S. cerevisiae* adaptive response. The transcription factor was identified as a functional homologue of mammalian AP-1 on the basis of its ability to bind to an AP-1 recognition element (Harshman *et al.*, 1988). Later studies showed that yap1 null mutants are hypersensitive to oxidative stress (Schnell *et al.*, 1992). Expression analysis of individual genes showed that Yap1p regulates the expression of several genes whose products play major roles in the oxidative stress tolerance. These targets include GSH1 encoding γ-glutamylcysteine synthetase (Wu and Moye-Rowley, 1994; Stephen *et al.*, 1995), GLR1 encoding glutathione reductase (Grant *et al.*, 1996), GPX2 encoding glutathione peroxidase (Inoue *et al.*, 1999), TRX2 encoding thioredoxin 2 (Kuge and Jones, 1994; Morgan *et al.*, 1997), TRR1 encoding thioredoxin reductase (Charizanis *et al.*, 1999; Lee *et al.*, 1999a), and the TSA1- and AHP1-encoded thioredoxin peroxidases (Lee *et al.*, 1999b). Whole-genome expression analysis by two-dimensional protein gels (Lee *et al.*, 1999a) and DNA microarrays (Gasch *et al.*, 2000) showed that Yap1p regulates the expression of many more genes in response to treatment with H2O2 as well as the superoxide-generating compound menadione and the thiol-oxidant diamide. A Yap1p recognition element was defined by studies of Yap1p binding to the TRX2 (5′-TTAG/CTAA) (Kuge and Jones, 1994) and GSH1 (5′-TTAGTCA) (Wu and Moye-Rowley, 1994) promoters and is present at many of the promoters of genes whose expression is induced in a Yap1p-dependent fashion.

The amino-terminus of Yap1p contains a bZip DNA-binding domain, which is conserved among the AP-1 family of proteins, including the mammalian cJun and cFos and *S. cerevisiae* Gcn4p (Moye-Rowley *et al.*,...
A carboxy-terminal cysteine-rich domain (c-CRD), containing three Cys–Ser–Glu repeats, was found to be critical for Yap1p-dependent gene activation and resistance to H\textsubscript{2}O\textsubscript{2} and diamide (Kuge et al., 1997; Wemmie et al., 1997). A second, more amino-terminal, cysteine-rich domain (n-CRD), also containing three cysteine residues, is only required for resistance to H\textsubscript{2}O\textsubscript{2} (Coleman et al., 1999). There is no increase in Yap1p protein levels and only a modest increase in Yap1p DNA-binding activity in response to oxidative stress (Kuge et al., 1997). Instead, the localization of the transcription factor changes dramatically (Kuge et al., 1997). In the absence of stress, Yap1p is present in both the cytoplasm and the nucleus. Upon treatment with H\textsubscript{2}O\textsubscript{2} and diamide, the protein is concentrated in the nucleus. Recent studies have shown that Yap1p localization is controlled by Crm1p-mediated nuclear export and that there are nuclear export signals within both the n-CRD and the c-CRD (Kuge et al., 1998; Yan et al., 1998; Coleman et al., 1999). Site-directed mutagenesis has indicated that cysteines within both the n-CRD and the c-CRD are critical for resistance and for the appropriate subcellular localization of Yap1p after oxidative stress (Kuge et al., 1998; Yan et al., 1998; Coleman et al., 1999). These results raise the intriguing possibility that the cysteines serve as redox sensors that regulate the accessibility of the nuclear export signals.

To gain insight into the regulation of Yap1p transcriptional activity, we carried out a genetic screen designed to identify new genes involved in the Yap1p regulatory pathway. This genetic screen allowed us to isolate genes conferring induced expression to the Yap1p-dependent thioredoxin 2 (TRX2) gene in the absence of oxidative stress. Here, we report the isolation of mutations in the TRR1 gene encoding thioredoxin reductase 1 and the effects of the trr1 mutations on the expression of Yap1p targets.

**Results**

**Isolation of mutants with induced TRX2 expression in the absence of H\textsubscript{2}O\textsubscript{2}**

The TRX2 gene, which encodes thioredoxin 2, is strongly induced by H\textsubscript{2}O\textsubscript{2} in a Yap1p-dependent manner (Kuge and Jones, 1994). To isolate mutants with altered Yap1p-dependent expression of TRX2, we constructed a fusion between the TRX2 promoter and the HIS3 gene. The promoterless HIS3 gene and the TRX2–HIS3 fusion were then integrated into haploid YPH499 cells that carry a chromosomal HIS3 deletion, generating YRS92 and YRS94 respectively. Residual expression from the promoterless HIS3 gene was sufficient to allow both strains to grow on SC minimal medium lacking histidine (SC–His). However, YRS92 did not grow on SC–His containing either 10 mM or 50 mM concentrations of the His3p inhibitor 3-amino-1,2,4-triazole (3-AT), and YRS94 only showed some residual growth in the presence of 50 mM 3-AT inhibitor (Fig. 1). Consistent with H\textsubscript{2}O\textsubscript{2}-dependent induction of TRX2 expression, YRS94 cells showed strong growth on SC–His plates containing 0.4 mM H\textsubscript{2}O\textsubscript{2} in addition to 50 mM 3-AT (data not shown).

To isolate mutants that constitutively express the TRX2–HIS3 fusion, YRS94 was mutagenized with methanesulphonatic acid ethyl ester (EMS) and plated on SC–His containing 75 mM 3-AT. Two mutants, YRS94#4 and YRS94#11, which showed strong growth in the presence of 50 mM and 75 mM 3-AT in the absence of H\textsubscript{2}O\textsubscript{2} (Fig. 1), were chosen for further study.

To determine whether the ability of YRS94#4 and YRS94#11 to grow on the SC–His medium in the presence of 75 mM 3-AT resulted from a cis-acting mutation at the TRX2 promoter fused to the HIS3 gene or from a trans-acting mutation, we examined the expression of the endogenous TRX2 gene by Northern blots (Fig. 2A). In the absence of H\textsubscript{2}O\textsubscript{2}, the TRX2 mRNA levels were elevated in YRS94#4 and YRS94#11 mutant cells compared with wild-type cells grown in YPD (Fig. 2A, lanes 3 and 5 compared with lane 1). Upon a 30 min exposure to 0.1 mM H\textsubscript{2}O\textsubscript{2}, TRX2 expression was further induced in the wild-type cells (Fig. 2A, lane 2) and in the YRS94#4 (Fig. 2A, lane 4) and YRS94#11 (Fig. 2A, lane 6) mutant cells. On average, compared with YRS94, the basal levels of the TRX2 mRNA were threefold higher in YRS94#4 and fourfold higher in YRS94#11. TRX2 expression was induced threefold for YRS94 and YRS94#4 and twofold for YRS94#11 upon treatment with H\textsubscript{2}O\textsubscript{2}.

Similar results were obtained by monitoring β-galactosidase activity in cells transformed with a plasmid containing the lacZ reporter gene under the control of the TRX2 promoter (Fig. 2B). In the absence of H\textsubscript{2}O\textsubscript{2}, the basal β-galactosidase activity of the YRS94#4 and YRS94#11 mutants was significantly higher than the basal activity of the wild-type strain (Fig. 2B). Upon a 30 min exposure to 0.1 mM H\textsubscript{2}O\textsubscript{2}, the wild-type and mutant strains showed increases in β-galactosidase activity. A mTRX2–lacZ plasmid carrying mutations in the Yap1p binding sites of the TRX2 promoter was not expressed in either wild-type or mutant cells, indicating that the enhanced TRX2 basal expression is Yap1p dependent (Fig. 2B). Taken together, these results suggest that the mutation leading to elevated expression of the TRX2–HIS3 and TRX2–lacZ fusions and the endogenous TRX2 gene is located in a trans-acting regulatory element.

The basal TRX2 mRNA levels in the mutants are approximately equal to the induced TRX2 mRNA levels found in the wild-type strain. In contrast, the basal
-galactosidase levels in the mutants are higher than the induced -galactosidase levels in the wild-type strain. We also observed significantly more induction of the TRX2 mRNA levels than of the -galactosidase activity by H2O2 in YRS94#4 and YRS94#11. These discrepancies between the Northern blots and the -galactosidase assays may result from the greater stability of the -galactosidase protein compared with the TRX2 mRNA.

To characterize the YRS94#4 and YRS94#11 mutant strains further, we also tested their sensitivity to H2O2 using a zone inhibition assay. Surprisingly, despite the elevated basal TRX2 mRNA levels, both mutants were found to be more sensitive to 2.5 M H2O2 than the wild-type strain (Table 1).

The MATa mutant strains also were mated with the MATa wild-type strain BY4709 to determine whether the mutation(s) in YRS94#4 and YRS94#11 are dominant or recessive. The heterozygous diploids had both normal basal and induced TRX2 expression (data not shown) as well as wild-type sensitivity to H2O2, indicating that the YRS94#4 and YRS94#11 mutation(s) are recessive and should be complemented by the intact genes from a wild-type genomic library.

Table 1. H2O2 sensitivity of YRS94 parent and mutant strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zones of inhibition (mm) with 2.5 M H2O2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>YRS94</td>
<td>23 23</td>
</tr>
<tr>
<td>YRS94#4</td>
<td>57 24</td>
</tr>
<tr>
<td>YRS94#11</td>
<td>58 25</td>
</tr>
<tr>
<td>YRS94 Δtrr1</td>
<td>55 26</td>
</tr>
</tbody>
</table>

a. Total diameter of the growth inhibition zone caused by the addition of H2O2. The values are from a representative assay.
Identification of the gene mutated in YRS94#4 and YRS94#11

To identify the mutation(s) involved in the constitutive expression of TRX2, an S. cerevisiae genomic library was transformed into mutant YRS94#4. Transformants were screened by replica plating onto plates containing 1, 2 and 4 mM H₂O₂. Plasmids isolated from colonies that grew on 2 and/or 4 mM H₂O₂ were retransformed into YRS94#4 and YRS94#11 and tested for complementation of the parental H₂O₂ sensitivity. Fourteen plasmids allowed YRS94#4 to grow in the presence of 2 mM H₂O₂ and conferred wild-type growth in a zone of inhibition assay (data not shown). These same plasmids also complemented YRS94#11 (data not shown), suggesting that the two strains carry mutations in the same gene(s).

The 14 complementing plasmids corresponded to one of two types of plasmids carrying an overlapping region of chromosome IV. Twelve had a 5.6 kb insert in one orientation, whereas two had a 7.9 kb insert in the opposite direction. The overlap of these two genomic fragments narrowed the region required for complementation down to position 1, 179, 342–1, 184, 927 on chromosome IV. Examination of the genes residing in this overlap region suggested that TRR1 might be the gene mutated in the two strains. Using polymerase chain reaction (PCR) with primers flanking the TRR1 gene, DNA fragments were generated from YRS94, YRS94#4 and YRS94#11 genomic DNA. The PCR product from each strain was sequenced directly revealing single point mutations in TRR1 in both YRS94#4 and YRS94#11 (Fig. 3). YRS94#4 carries a mutation of a G to an A at

Fig. 3. Position of mutations in YRS94#4 and YRS94#11. The thioredoxin reductase protein sequences of S. cerevisiae (AAB64789), S. pombe (CAJ17652), A. thaliana (Q399243) and E. coli (BA355620) were aligned using CLUSTAL W (1.8) software. Identical amino acid residues are indicated by the asterisks, the redox-active centre is indicated by the box, and the arrowheads point to the amino acid changes in YRS94#4 (G138D) and YRS94#11 (G243D).
position 413, resulting in the conversion of a glycine to an aspartic acid at position 138. YRS94#11 carries a mutation of a G to an A at position 728, resulting in the conversion of a glycine to an aspartic acid at position 243. The TRR1 gene encodes the 34 kDa thioredoxin reductase protein that catalyses the reduction of oxidized thioredoxin at the expense of NADPH. The deduced amino acid sequence of Trr1p is 49% identical to the Escherichia coli and 61% identical to the Arabidopsis thaliana thioredoxin reductases whose structures have been solved (Dai et al., 1996; Lennon et al., 1999). The amino acids comprising the thioredoxin active site as well as the NADPH and FAD binding sites are conserved with the E. coli, A. thaliana and Schizosaccharomyces pombe homologues. The two conserved glycines that are mutated in YRS94#4 and YRS94#11 are both near the CAVC active site in the three-dimensional structures of the bacterial and plant homologues.

Effect of Δtrr1 mutation on TRX2 expression

To examine the role of TRR1 in the Yap1p-dependent regulation of gene expression, we constructed a Δtrr1 strain by replacing the entire TRR1 open reading frame (ORF) in YRS94 with the kanMX4 gene cassette. Consistent with a previous report (Machado et al., 1997), the YRS94 Δtrr1 mutant had reduced growth rates compared with the wild-type strain and was hypersensitive to H2O2 (Table 1). We also observed that the mutant was unable to grow on glycerol as a carbon source (data not shown). The YRS94 Δtrr1 strain was tested for TRX2 expression by Northern blot analysis (Fig. 4). Similar to the YRS94#11 mutant, YRS94 Δtrr1 exhibited an ∼fourfold higher basal level of TRX2 mRNA compared with YRS94 (Fig. 4, lane 3 compared with lane 1). The expression was further induced ∼twofold upon treatment with H2O2 (Fig. 4, lane 4). The basal and induced levels of β-galactosidase activity for the YRS94 Δtrr1 strain carrying the TRX2–lacZ fusion plasmid were almost identical to the levels observed for YRS94#11 (data not shown). Although it is formally possible that the YRS94#4 and YRS94#11 strains contain recessive mutations in genes other than TRR1, the similarities in the YRS94#4, YRS94#11 and YRS94 Δtrr1 phenotypes indicate that the defects reported for YRS94#4 and YRS94#11 result from the inactivation of thioredoxin reductase.

The wild-type TRR1 gene was also cloned into a yeast centromeric plasmid to generate pYOH8. YRS94#4 and YRS94#11 transformed with pYOH8 had basal and induced TRX2 expression similar to those measured in the wild-type strain (Fig. 4) and showed wild-type resistance to H2O2 (Table 1). These results verify that the YRS94#4 and YRS94#11 mutant phenotypes are a result of at least partial loss of thioredoxin reductase function.

Effect of Δtrr1 mutation on global gene expression

Whole-genome expression analysis has proved to be a powerful tool in examining the global effects of specific mutations and specific environmental conditions (reviewed by Brown and Botstein, 1999). In yeast, this technique allows every ORF in the genome to be analysed for differences in mRNA transcript levels when a pair of strains or culture conditions is compared. Hierarchical clustering of gene expression data organizes genes according to similar expression profiles and indicates which genes are co-regulated (Eisen et al., 1998). In addition, the results of these assays can be compared with the large collection of array experiments carried out with isogenic strains.

To gain more insight into the effects of the Δtrr1 deletion, we used DNA microarrays to compare the whole-genome expression patterns of wild-type (DBY7286) and Δtrr1 mutant (DBY7286 Δtrr1) cells left untreated or treated with 0.1 and 0.3 mM H2O2. Figure 5A shows a cluster diagram of the expression of genes previously identified as potential Yap1p targets (Gasch et al., 2000). The data are shown for DBY7286 Δtrr1 versus untreated DBY7286 (Fig. 5A, column 1), DBY7286 treated with 0.3 mM H2O2 versus untreated DBY7286 (Fig. 5A, column 2) (Gasch et al., 2000), DBY7286 Δyap1 treated with 0.3 mM H2O2 versus untreated DBY7286...
Constitutive Yap1p activation in trr1 mutants

Δyap1 (Fig. 5A, column 3) (Gasch et al., 2000) and untreated DBY7286 with a YAP1-overexpressing vector versus untreated DBY7286 with a control vector (Fig. 5A, column 4) (DeRisi et al., 1997). Genes that are expressed at higher levels in the Δtrr1 deletion in response to H2O2 treatment and after YAP1 overexpression are indicated as red boxes according to the depicted scale. This comparison showed that, in the Δtrr1 mutant strain, >70% of the putative Yap1p-induced genes are expressed at basal levels that are ≥1.5-fold higher than the basal levels in the wild-type strain in the absence of H2O2 treatment. Interestingly, other H2O2-inducible stress genes, many of which are targets of the transcription factors Msn2p and Msn4p but not Yap1p (such as CTT1 and HSP12), are also expressed at higher basal levels in untreated DBY7286 Δtrr1 (data not shown). Together, these results suggest that, under standard growth conditions, the Δtrr1 mutant experiences stress similar to that induced in cells by the presence of exogenously added H2O2, possibly because of increased internal levels of ROS and/or alterations in the cellular redox potential.

We also compared the expression of genes that are induced or repressed ≥twofold in Δtrr1 mutants treated with 0.1 and 0.3 mM H2O2 (Fig. 5B, columns 2 and 4) with the expression of the corresponding genes in the wild-type cells treated with 0.1 and 0.3 mM H2O2 (Fig. 5B, columns 1 and 3). The comparison showed that ≥50% of the putative Yap1p targets are induced ≥1.5-fold more strongly by 0.1 and 0.3 mM H2O2 in DBY7286 Δtrr1 compared with DBY7286. In a previous study, we discovered a large set of ~900 genes, termed the environmental stress response (ESR), that are similarly activated or repressed by a large variety of environmental insults including heat shock, oxidative stress, osmotic shock and starvation (Gasch et al., 2000). On average, ESR genes were induced or repressed twofold more strongly in the H2O2-treated Δtrr1 cells than in the untreated Δtrr1 and H2O2-treated wild-type cells. We suggest that the effects of exogenously added H2O2 are more severe in the Δtrr1 mutant as a result of the diminished capacity of the mutant cells to detoxify H2O2 or other oxidants and/or to repair oxidative-stress-induced damage. However, we did not observe a significant difference in viability between the wild type (95%) and the Δtrr1 mutant (92%) 30 min after exposure to the low 0.3 mM concentration of H2O2.

We also found that genes encoding multiple classes of protein-folding chaperones are strongly induced in treated DBY7286 Δtrr1 but not in the treated wild-type or the untreated Δtrr1 mutant strains. The hyperinduced chaperone genes include members of the Hsp70 family (such as SSE1 and SSA2), the Hsp90 family (such as HSP82, STI1 and CPR6), the Hsp10/60 family and the ESR group of chaperones (such as HSP42, HSP104, HSP78, SSA3 and SSA4). The hyperinduction of these genes, which are generally required under conditions that generate denatured proteins (Morano et al., 1998), suggests that Δtrr1 mutants treated with H2O2 have increased levels of misfolded proteins compared with treated wild-type cells.

Discussion

Previous studies have shown that the Yap1p transcription factor is a major regulator of the S. cerevisiae response to H2O2. To identify factors that regulate Yap1p activity, we carried out a genetic screen for mutants that show elevated expression of a TRX2–HIS3 fusion in the absence of H2O2. Two independent strains recovered in our screen carried mutations in the TTRR1 gene encoding thioredoxin reductase. Whole-genome expression analysis revealed that the basal expression of Yap1p targets is elevated in Δtrr1 mutants and that the Yap1p targets are induced more strongly by H2O2 in Δtrr1 mutants than in the wild-type strain. Our results, together with the finding that strains lacking both thioredoxin 1 and thioredoxin 2 (trx1 trx2 mutants) have constitutive Yap1p activity in the absence of stress (Izawa et al., 1999), strongly implicate the thioredoxin system, consisting of thioredoxins and thioredoxin reductase, in modulating the activity of the Yap1p transcription factor.

Fig. 5. Effect of TTRR1 deficiency on global gene expression patterns. Cultures in early log phase (OD600 = 0.4) were split and grown in the absence or the presence of 0.1 mM or 0.3 mM H2O2 for 30 min.
A. Poly A+ RNA was isolated from untreated DBY7286 and DBY7286 Δtrr1, labelled and hybridized to microarrays. The expression profile was then compared with the expression profiles of DBY7286 and DBY7286 Δyap1 treated with 0.3 mM H2O2 (Gasch et al., 2000) and DBY7286 overexpressing Yap1p (DeRisi et al., 1997). Column 1 corresponds to untreated DBY7286 Δtrr1 versus untreated DBY7286; column 2 corresponds to DBY7286 treated with 0.3 mM H2O2 versus untreated DBY7286; column 3 corresponds to DBY7286 Δyap1 treated with 0.3 mM H2O2 versus untreated DBY7286 Δyap1; and column 4 corresponds to untreated DBY7286 overexpressing Yap1p versus untreated DBY7286. Grey boxes denote missing data. A plus sign (+) to the left of the columns indicates genes that contain ≥1 putative Yap1p binding site (5’-TTAG/CTAA or 5’-TTAGTCA) within 1000 bp of the initiating codon, and an asterisk in the gene annotation indicates genes likely to cross-hybridize. The gene names and functions to the right of the columns are taken from http://genome-www.stanford.edu/Saccharomyces.
B. Poly A+ RNA was isolated from untreated and treated DBY7286 and DBY7286 Δtrr1, labelled and hybridized to microarrays. Column 1 corresponds to DBY7286 treated with 0.1 mM H2O2 versus untreated DBY7286; column 2 corresponds to DBY7286 Δtrr1 treated with 0.1 mM H2O2 versus untreated DBY7286 Δtrr1; column 3 corresponds to DBY7286 treated with 0.3 mM H2O2 versus untreated DBY7286; column 4 corresponds to DBY7286 Δtrr1 treated with 0.3 mM H2O2 versus untreated DBY7286 Δtrr1; and column 5 corresponds to untreated DBY7286 Δtrr1 versus untreated DBY7286. The experiment was repeated twice, and the complete data set is available at http://www-genome.stanford.edu/trr1.

© 2001 US Government, Molecular Microbiology, 39, 595–605
There are several possible models for the effects of the *trr1* and *trx1 trx2* mutations. The lack of thioredoxin reductase or thioredoxin 1 and 2 might result in decreased ability of the mutant cells to detoxify H$_2$O$_2$ or other oxidants. For example, the absence of reduced thioredoxin will impair the ability of the thioredoxin-dependent peroxidases, encoded by *TSA1*, *AHP1*, *YDR453C*, *YBL064C* and *YIL010W*, to remove peroxides from the cell. In this model, increased peroxide levels would lead to the oxidation of Yap1p itself or another, as yet unidentified, redox-sensitive protein that modulates Yap1p activity (Fig. 6A). Alternatively, the thioredoxin system might be acting to reduce Yap1p or the hypothesized redox-sensitive protein. In this model, Yap1p or the putative Yap1p regulator would be oxidized by the peroxides present in the cell in the absence of external stress, but the oxidized protein would not be efficiently reduced, leading to the accumulation of activated Yap1p (Fig. 6B). A combination of the two models is also likely, and both models are consistent with the results of the microarray experiments.

The two models for the effects of the *trr1* mutation may also explain the conundrum that Δ*trr1* mutants are hypersensitive to H$_2$O$_2$ despite the elevated levels of many antioxidant activities. Although the expression of the thioredoxin-dependent peroxidases is elevated, the enzymes may not be able to remove peroxides without reduction by the thioredoxin system. The accumulation of proteins with abnormal disulphide bonds may also contribute to the increased H$_2$O$_2$ sensitivity. Regardless of the model, an important conclusion from our study is that strains with elevated expression of genes encoding antioxidant enzymes may still be hypersensitive to oxidative stress.

The reducing environment of the cell is maintained by both the thioredoxin system and the glutaredoxin system, consisting of glutaredoxins, glutathione and glutathione reductase. In *E. coli*, the activity of the redox-sensitive OxyR transcription factor is primarily modulated by the glutaredoxin system (Zheng et al., 1998). Our results and the findings that Yap1p is constitutively active in *trx1 trx2* mutants implicate the thioredoxin system in modulating Yap1p activity in *S. cerevisiae*. Izawa et al. (1999) found that a deficiency in *S. cerevisiae* glutaredoxin 1 and glutaredoxin 2 (*grx1 grx2*) does not lead to the constitutive activation of Yap1p and suggested that the glutaredoxin system does not modulate Yap1p activity. The lack of a *grx1 grx2*-dependent Yap1p phenotype might be explained by the presence of three additional glutaredoxin proteins present in yeast. However, we have found that a deletion of the single gene encoding glutathione reductase (*glr1*) also does not affect Yap1p activity (data not shown). It is not clear why OxyR activity is regulated by the glutaredoxin system whereas Yap1p activity is regulated by the thioredoxin system, but it will be interesting to see whether the activities of other transcription factors are modulated specifically by one or the other reducing system.

Our genetic screen has defined the *TRR1*-encoded thioredoxin reductase as an activity that is important for the redox regulation of Yap1p. It is likely that yet other proteins are involved. Our *TRX2–HIS3* screen was not saturated and may reveal additional activities that modulate Yap1p activity. It will also be interesting to carry out biochemical analysis to determine whether Yap1p is oxidized directly and, if so, whether thioredoxin 1 or thioredoxin 2 reduce Yap1p in vivo.

**Experimental procedures**

**Yeast strains, growth conditions and techniques**

The strains and plasmids used in this study are listed in Table 2. YPH499 was the wild-type strain for all genetic experiments, BY4709 was used in the mating experiments, and DBY7286 and its derivative DBY7286 Δ*trr1* were used in the microarray experiments. *S. cerevisiae* strains were grown in either YPD rich media (Sherman, 1991) for non-selective growth or drop-out SC minimal media (Bio101) for selective growth. Yeast cells were transformed using the lithium acetate–PEG method as described previously (Gietz and Schiestl, 1995).
Construction and mutagenesis of YRS94

The promoter region upstream of the TRX2 (YGR209C) gene (from chromosome VII position 913, 508–913, 222) was PCR amplified (Trx2-Prtm1A (5′-CGACTTCTAGATCGACTTAUGTTG1TGCAGTG) and Trx2-Prtm1B (5′-CGA CTGGAACAGAGCCCTTTAGATCTGTAAGTAAGATCTGAC); Xbal and BamHI restriction sites are underlined) from genomic S. cerevisiae DNA isolated from the wild-type strain F113. The promoter fragment included the complete intergenic region between the upstream ORF (YGR210C) and the –1 nucleotide from the Trx2p initiating methionine. PCRs using 5 units of Pfu polymerase (Stratagene), 0.35 μg of genomic DNA, 0.25 mM dNTPs and 0.5 mM primers per 100 μl reaction volumes were run in a preheated thermal cycler at 94°C for 3 min, followed by five cycles at 94°C for 30 min, 62°C for 30 min, 68°C for 60 min and 20 cycles at 94°C for 30 min, 56°C for 30 min, 68°C for 60 min. The reaction products were purified using Qia-Quick columns (Qiagen), digested with Xbal and BamHI and cloned into the corresponding sites (dephosphorylated) of the yeast integrating plasmid pCM105, which contains a promoterless HIS3 gene and URA3 as a selectable marker. The sequence of the pPTRX2-HIS3 clone was verified on both strands before introduction into yeast. YPH499 was transformed with Ncol-digested pPTRX2-HIS3 with selection for uracil prototrophy. YRS94 (1 × 10⁸ cells ml⁻¹) was mutagenized with EMS (20 μl; Sigma) in two separate mutagenesis reactions. A total of ~2 × 10⁸ cells were plated on SC–His supplemented with 75 mM 3-AT (Fluka). In addition to inhibiting His3p, 3-AT has been reported to inhibit yeast catalases (Kowaltowski et al., 2000) and possibly contributed to oxidative stress on the selection plates. About 50 colonies that grew after 4 days at 30°C were rescreened. Two mutants that grew well on SC–His + 75 mM 3-AT plates and in YPD liquid culture and had altered sensitivity to H₂O₂ were chosen for further characterization.

Table 2a. Yeast strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH499</td>
<td>Sikorski and Hieter (1989)</td>
<td>MATα ura3-52 lys2-801sup* ade2-101::trp1-Δ63 his3-Δ200 leu2-Δ1</td>
</tr>
<tr>
<td>YRS92</td>
<td>This study</td>
<td>YPH499 URA3::pCM105</td>
</tr>
<tr>
<td>YRS94</td>
<td>This study</td>
<td>YPH499 URA3::pPTRX2-HIS3</td>
</tr>
<tr>
<td>YRS94 Δtrr1</td>
<td>This study</td>
<td>YRS94 trr1-Δ0</td>
</tr>
<tr>
<td>DBY7286</td>
<td>Spellman et al. (1998)</td>
<td>DBY7286 GAL2 ura3</td>
</tr>
<tr>
<td>DBY7286 Δtrr1</td>
<td>This study</td>
<td>DBY7286 trr1-Δ0</td>
</tr>
<tr>
<td>F113</td>
<td>Laboratory stock</td>
<td>MATα, can1, ino1-13, ura3-52</td>
</tr>
<tr>
<td>BY4709</td>
<td>Brachmann et al. (1998)</td>
<td>MATα ura3-Δ0</td>
</tr>
</tbody>
</table>

Table 2b.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCM105</td>
<td>Laboratory stock</td>
<td>A derivative of pCM99 (Dancis et al., 1994) containing a modified multicloning site at the 5′ side of the HIS3 reporter gene</td>
</tr>
<tr>
<td>pPTRX2-HIS3</td>
<td>This study</td>
<td>TRX2 promoter cloned into the Xbal and BamHI sites of pCM105</td>
</tr>
<tr>
<td>pYOH8</td>
<td>This study</td>
<td>TRR1 cloned into the HindIII and Pst sites of pRS415 (Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pYOH10</td>
<td>This study</td>
<td>The 4.2 kb Ncol TRX2–lacZ fragment (filled in) from TRXLACZ (Kuge and Jones, 1994) cloned into the SmaI site of pRS415</td>
</tr>
<tr>
<td>pYOH11</td>
<td>This study</td>
<td>The 4.2 kb Ncol TRX2–lacZ fragment (filled in) from mTRXLACZ (Kuge and Jones, 1994) cloned into the SmaI site of pRS415</td>
</tr>
</tbody>
</table>

Complementation of YRS94#4

An S. cerevisiae genomic library (ATCC 37323) in YEp13 was transformed into YRS94#4 with selection for leucine prototrophy. The ~125 000 transformants (> 10-fold representation of the yeast genome) were screened by replica plating onto SC-Leu containing 1 mM H₂O₂. Approximately 650 large colonies were observed with a high background of smaller colonies. As a control, transformants were also replicated on SC–Leu–Trp, and roughly 650 colonies were obtained without a background of smaller colonies. The large colonies identified on the SC–Leu + 1 mM H₂O₂ plates were replicated onto plates with 2 or 4 mM H₂O₂. Plasmids isolated from 16 of the colonies that grew on 2 mM and/or 4 mM H₂O₂ were retransformed into YRS94#4 and tested for complementation of the parental H₂O₂ sensitivity. Fourteen plasmids restored wild-type resistance to H₂O₂. The remaining two plasmids did not restore wild-type growth and were therefore probably false positives.

Sequencing and cloning of TRR1

The TRR1 gene (from –611 to 1557) was PCR amplified (TRR1-F1 (5′-GCGATTAAGCTTAGCGACACCTAGTGACAGACG) and TRR1-R1 (5′-GCGATCTGGACATATTACCCAGACGGATCTAGTGGAG); HindIII and Pst restriction sites are underlined) from genomic S. cerevisiae DNA isolated from strains YRS94, YRS94#4 and YRS94#11. The pool of PCR products for each strain was sequenced directly. In addition, the purified PCR fragment from YRS94 was digested with HindIII and Pst and cloned into the corresponding restriction sites of pRS415 to generate pYOH8.

TRR1 gene disruptions

Gene deletions were performed by the one-step PCR-based gene disruption technique (Wach, 1996). A 1.6 kb PCR product was amplified using primer pairs 0436.0F (5′-CGTGGCGTGCGTCGACGACG; HindIII site) and 0436.0R (5′-CAAGGAGGGGCCACGGACGACG; Pst site). The PCR fragment was digested with HindIII and Pst and cloned into the SmaI site of pRS415 to generate pYOH8. The plasmid was cotransformed with pCM105 into YPH499 to create DBY7286. Deletions were confirmed by PCR and Southern blot analysis of the genomic DNA isolated from DBY7286. The deletion constructs were then transformed into YRS94 and screened for the loss of the HIS3 marker.

© 2001 US Government, Molecular Microbiology, 39, 595–605
fragment containing the kanMX4 gene was PCR amplified [TRR1-A (5'-GCTATACGCAAATAGCACTAGCAGAA GTCACATCATTTATTGAGTACCAGTACGGTCGG) and TRR1-B (5'-TGGATAATGCTACCAATCGATGAAATCGGTGACCTG)], kanMX4 sequence is underlined] from pFA6a (Wach et al., 1994) and then transformed into YRS94 and DBY7286 with selection for G418 (Life Technologies) resistance. Deletions were confirmed by PCR using the appropriate primers directed against the kanMX4 region as well as sequences flanking the TRR1 gene.

Northern blot analysis

Yeast cells from overnight cultures were diluted to OD_{600} of 0.05 in the appropriate medium and incubated with shaking at 30°C until they reached an OD_{600} of 0.4. The cells were then aliquoted and incubated in the absence or presence of H_{2}O_{2} (0.1 mM) for 30 min. Total RNA was prepared by the hot phenol method (Schmitt et al., 1990), followed by treatment with Trizol reagent according to the manufacturer’s protocol (Life Technologies). The RNA pellet was resuspended in 10 mM Tris- HCl, pH 7.4. Aliquots (10 μg) of total RNA for each tested condition were separated by electrophoresis on a 1.4% agarose–formaldehyde gel, transferred to Nitran Super- Charge membrane (Schleicher and Schuell) and hybridized with 32P-labelled DNA-specific probes. TRX2 transcripts were probed using a 32P end-labelled oligonucleotide (5'-TACCG GAACATGCTGGTGCGTA-3'). A DNA fragment generated by PCR from the ORF of ACT1 [ACT1-1 (5'-TCGGCTGAC GTACCGCTGCAGGTCGAC) and OCH-212 (5'-TGGAAA CATGGTGG) was labelled using the Prime-It II random primer labelling kit (Stratagene). Hybridization with the ACT1 probe served as a control for RNA loading. The intensities of the TRX2 and ACT1 bands were quantified on a phosphorimager (Molecular Dynamics) to calculate the expression levels.

Microarrays

The growth conditions and procedure for total RNA extraction were as described above for the Northern analysis. Poly A+ mRNA was isolated using oligotex beads (Qiagen) according to the manufacturer’s instructions. Probe preparation, microarray production, hybridization and data analysis were performed as described previously (DeRisi et al., 1997; Spellman et al., 1998; Gasch et al., 2000). The complete data set is available at http://www-genome.stanford.edu/trr1. Quantification of the induction of the Yap1p target genes was carried out by comparing individual data points. Quantification of the induction of the ESR response was carried out by averaging the values for the wild type and the Δtrr1 mutant and then comparing the averages.

Sensitivity assay

Filter disks (3 mm; Whatman) soaked with 10 μl of 1.0, 2.5 or 5.0 M H_{2}O_{2} were placed on lawns made by plated 100 μl of early log phase cells (OD_{600} = 0.4). The zones of inhibition around the filter disks were measured after 2 days of incubation at 30°C.

β-Galactosidase assay

Cells (1 x 10^7) were harvested in an Eppendorf tube, washed once with water, resuspended in 800 μl of Z buffer (60 mM Na_{2}HPO_{4}, 40 mM NaH_{2}PO_{4}, 10 mM KCl, 1 mM MgSO_{4} and 50 mM 2-mercaptoethanol, pH 7.0) and placed on ice. Cells were permeabilized by the addition of 10 μl of 0.1% SDS and 20 μl of chloroform. β-Galactosidase activity was determined by mixing the cell extract with a stock solution (4 mg ml^{-1}) of ONPG and incubating the mixture at 30°C for 20–30 min. The reaction was then quenched by adding 400 μl of 1 M sodium carbonate. Unbroken cells and cell debris were removed by centrifugation for 2 min, and colour intensity was measured. The activity was calculated as Miller units = [OD_{420} × 1000]/[OD_{600} × time (min) × volume (ml)].

Acknowledgements

We thank S. Kuge and Dr. P. Philippsen for plasmids. We also appreciate the editorial comments of F. Aslund, J. Fassler and M. Zheng. This work was supported by the intramural programme of the National Institute of Child Health and Human Development (O.C.-H. and G.S.), a fellowship from the European Molecular Biology Organization (O.C.-H.) and grants from the National Institutes of Health (D.B. and P.O.B.). P.O.B. is an associate investigator of the Howard Hughes Medical Institute.

References


