Transcriptional Activation of Metalloid Tolerance Genes in *Saccharomyces cerevisiae* Requires the AP-1–like Proteins Yap1p and Yap8p

Robert Wysocki, Pierre-Karl Fortier, Ewa Maciaszczyk, Michael Thorsen, Anick Leduc, Åsa Ödhagen, Grzegorz Owsianik, Stanislaw Ulaszewski, Dindial Ramotar, and Markus J. Tamás

All organisms are equipped with systems for detoxification of the metalloids arsenic and antimony. Here, we show that two parallel pathways involving the AP-1–like proteins Yap1p and Yap8p are required for acquisition of metalloid tolerance in the budding yeast *S. cerevisiae*. Yap8p is demonstrated to reside in the nucleus where it mediates enhanced expression of the arsenic detoxification genes *ACR2* and *ACR3*. Using chromatin immunoprecipitation assays, we show that Yap8p is associated with the *ACR3* promoter in untreated as well as arsenic-exposed cells. Like for Yap1p, specific cysteine residues are critical for Yap8p function. We further show that metalloid exposure triggers nuclear accumulation of Yap1p and stimulates expression of antioxidant genes. Yap1p mutants that are unable to accumulate in the nucleus during H₂O₂ treatment showed nearly normal nuclear retention in response to metalloid exposure. Thus, our data are the first to demonstrate that Yap1p is being regulated by metalloid stress and to indicate that this activation of Yap1p operates in a manner distinct from stress caused by chemical oxidants. We conclude that Yap1p and Yap8p mediate tolerance by controlling separate subsets of detoxification genes and propose that the two AP-1–like proteins respond to metalloids through distinct mechanisms.

**INTRODUCTION**

Exposure to the toxic metalloids arsenic and antimony is a serious challenge to all organisms. In humans, arsenic compounds are associated with an increased incidence of a variety of diseases, including cancer. Yet, metalloid-containing drugs are used as chemotherapeutic agents to combat infectious diseases caused by pathogenic parasites as well as cancer, including acute promyelocytic leukemia (Murray, 2001; Waxman and Anderson, 2001). The emergence of metalloid tolerance is a considerable threat to effective medical treatment and makes the elucidation of the mechanisms that form the basis of tolerance a high priority (Tamás and Wysocki, 2001).

A number of proteins involved in metalloid transport and tolerance have been described in various organisms. In the eukaryotic model organism *Saccharomyces cerevisiae* (bakers' yeast) two transport systems contribute to metalloid removal from the cytosol, Acr3p, and Ycf1p. Acr3p is a plasma membrane protein that extrudes As(III) from the cell (Wysocki et al., 1997; Ghosh et al., 1999), whereas the ATP-binding cassette-transporter Ycf1p mediates uptake of glutathione-conjugates of As(III) and Sb(III) into the vacuole (Ghosh et al., 1999). Inactivation of ACR3 sensitizes cells to As(III) and As(V), whereas inactivation of *YCF1* causes As(III) and Sb(III) sensitivity (Wysocki et al., 1997; Ghosh et al., 1999; Wysocki et al., 2001). Because arsenic is removed from the cytosol in the form of As(III), yeast cells reduce As(V) to As(III) by the action of the cytosolic arsenate reductase *Acr2p* (Mukhopadhyay et al., 2000).

To date, little is known about metalloid-specific signal transduction and transcriptional regulation of detoxification genes. As(III) seems to stimulate transcription of various stress-responsive genes in mammals, probably via an AP-1 transcription factor (Del Razo et al., 2001). *S. cerevisiae* contains eight fungal-specific AP-1–like proteins: Yap1p to Yap8p. These proteins contain a bZIP DNA binding domain as well as conserved cysteine-rich domains (CRD) in their amino and carboxy termini (n-CRD and c-CRD, respectively) (Fernandes et al., 1997; Toone et al., 2001). Yap1p is crucial for oxidative stress tolerance in yeast; *YAP1* deletion results in hypersensitivity to peroxide, the thiol oxidant diamide, certain electrophiles, and cadmium (Toone et al., 2001). When cells are exposed to oxidants, Yap1p transiently accumulates in the nucleus (Kuge et al., 1997; Yan et al., 1998; Delaunay et al., 2000; Kuge et al., 2001) and activates transcription of genes coding for proteins that maintain a favorable cellular redox balance as well as for enzymes involved...
in detoxification of reactive oxygen species (ROS) (Lee et al., 1999; Gasch et al., 2000). In addition, Yap1p mediates cadmium tolerance by controlling YCF1 expression (Wemmie et al., 1994). Interestingly, different oxidants activate Yap1p in distinct ways; activation by H₂O₂ occurs through formation of an intramolecular disulfide bond between C₃₀₃ and C₅₉₈ (Delaunay et al., 2001; Toledano et al., 2002). Whether Yap1p oxidation by other stress agents also involves effector proteins has yet to be demonstrated.

Whereas Yap1p has been extensively studied, the physiological functions of the other yeast AP-1-like proteins are currently unknown, and data are largely restricted to overexpression phenotypes (Toone and Jones, 1999; Toone et al., 2000), whereas diamide activation involves the C-terminal cytoxines C₅₉₈, C₆₂₀, and C₆₂₉ (Kuge et al., 2001). It has recently been shown that the glutathione peroxide-like protein Gpx3p is required for Yap1p oxidation by H₂O₂ (Delaunay et al., 2002). Whether Yap1p oxidation by other stress agents also involves effector proteins has yet to be demonstrated.

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by using pGAL-GFP-Yap8p as template. The oligonucleotides used to create the C132A mutation were YAP8-C132A-A (5'-CTTTGCGTTCTGAAGAGAACTTTTCTCAGCAGGAGCTGCC-3') and YAP8-C132A-B (5'-GCACGCTGAATCTTCTCTTCAACAACCTCAGTGAAGACTGTCG-3'). The oligonucleotides used to create the C274A mutation as well as the double mutation C132A C274A were synthesized with centrally located changes underlined. Similarly, the oligonucleotides used to create the C274A /H11032 point mutation in pGAL-GFP-Yap8p (pYES2) were used to create the C274A /H11032 point mutation in pGAL-GFP-Yap8p (pYES2) which was digested with NcoI and XbaI, respectively.

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
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<tbody>
<tr>
<td>pUG6</td>
<td>Vector containing theloxP-kanMX-loxP deletion cassette</td>
<td>Guldener et al. (1996)</td>
</tr>
<tr>
<td>YEp357R</td>
<td>2μ vector with lacZ reporter gene, URA3</td>
<td>Myers et al. (1986)</td>
</tr>
<tr>
<td>YEp363</td>
<td>2μ vector with lacZ reporter gene, LEU2</td>
<td>Myers et al. (1986)</td>
</tr>
<tr>
<td>pSEY102</td>
<td>CEN vector with lacZ reporter gene, URA3</td>
<td>Emr et al. (1986)</td>
</tr>
<tr>
<td>GFP-YAP8p</td>
<td>pRS316 (CEN, URA3), Yap1p fused to GFP</td>
<td>Coleman et al. (1999)</td>
</tr>
<tr>
<td>pYES2</td>
<td>2μ vector with GALI promoter, URA3</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGAL-GFP-Yap8p</td>
<td>GFP fused to N terminus of YAP8 in pYES2</td>
<td>This work</td>
</tr>
<tr>
<td>pEM16</td>
<td>ACR2-lacZ fusion in YEp357R</td>
<td>This work</td>
</tr>
<tr>
<td>pEM19</td>
<td>ACR3-lacZ fusion in pSEY102</td>
<td>This work</td>
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<tr>
<td>pRW11</td>
<td>ACR3-lacZ fusion in YEp536</td>
<td>This work</td>
</tr>
<tr>
<td>pRW33</td>
<td>ACR3 behind its own promoter lacking the TTAATAA sequence</td>
<td>(mutACR3) on pRW3</td>
</tr>
<tr>
<td>pEM18</td>
<td>mutACR2-lacZ fusion lacking the TTAATAA sequence in YEp357R</td>
<td>This work</td>
</tr>
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<td>pEM20</td>
<td>mutACR3-lacZ fusion lacking the TTAATAA sequence in pSEY102</td>
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<td>GFP-YAP8-C132A</td>
<td>C132A point mutation in pGAL-GFP-Yap8p (pYES2)</td>
<td>This work</td>
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<tr>
<td>GFP-YAP8-C274A</td>
<td>C274A point mutation in pGAL-GFP-Yap8p (pYES2)</td>
<td>This work</td>
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<tr>
<td>pRS-cp-GFP HA YAP1</td>
<td>GFP-fused Yap1p (wt) in pRS314 (CEN, TRP1)</td>
<td>Kuge et al. (1997)</td>
</tr>
<tr>
<td>pRS-cp-GFP HA YAP1 cm46A5</td>
<td>GFP-fused Yap1p-TAT (C598T C620A C629T) in pRS314</td>
<td>Kuge et al. (1997)</td>
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<tr>
<td>pRS-cp-GFP HA YAP1 3Cys</td>
<td>GFP-fused Yap1p-3Cys (C304T C310T C315T) in pRS314</td>
<td>Kuge et al. (2001)</td>
</tr>
<tr>
<td>YEp159-GFP-YAP8</td>
<td>GFP-YAP8 controlled by the endogenous YAP8 promoter in YEplac195</td>
<td>This work</td>
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<td>pJAW96</td>
<td>YCF1-lacZ fusion in pSEY102</td>
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<td>pCM188</td>
<td>CEN vector, URA3, tetO-CYC1 promoter</td>
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<td>pM1004</td>
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<td>This work</td>
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</tbody>
</table>

Expression Analysis

Northern Blot. Sodium arsenite (1 mM) or potassium antimonyl tartrate (1 mM) was added to exponentially growing cells on YNB medium. Total RNA was isolated at the time points indicated in Figure 3 and separated on formamide-agarose gels by using standard methods. Blots were hybridized with 32P-labeled PCR fragments in buffer containing 7% SDS, 0.5 M sodium phosphate buffer, pH 7.0, and 1 mM EDTA. To quantify transcript levels, signal intensity was quantitated with a Molecular Dynamics Phosphoimager and normalized to 18S rRNA. At least two independent Northern blot analyses were performed for each growth condition and transcript examined.

β-Galactosidase Activity Measurements. Cells expressing the various lacZ fusion genes were grown in YNB glucose medium for 20 h in the presence of low concentration of metalloids: wild type [0.1 mM As(III), As(V), Sb(III)], yap1Δ [0.05 mM As(III), As(V); 0.1 mM Sb(III)], yap1Δ [0.05 mM As(III), Sb(III); 0.1 mM As(V)], yap1Δ yap8Δ [0.05 mM As(III), As(V), Sb(III)]. For the last 2 h of incubation, the concentration of metalloids was increased to 1 mM. β-Galactosidase activity assays were performed on permeabilized cells as described previously (Guarente, 1983) at least two times for three independent transformants. The values are given with SD.

Chromatin Immunoprecipitation (ChIP) Assays

Exponentially growing yeast cells expressing FLAG-tagged Yap8p were crosslinked with 1% formaldehyde, incubated with 125 mM glycine, harvested, and washed in phosphate-buffered saline. Cell breakage was performed in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1% protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN) by using glass bead grinding followed by sonication for 90 s to yield an average DNA fragment size of 500 bp. Immunoprecipitation was performed with agarose beads, which were coated with mouse anti-FLAG-IgG (Sigma Aldrich, St. Louis, MO) and incubated with anti-FLAG monoclonal antibody (M2; Sigma Aldrich) beforehand. Precipitates were washed and processed for DNA purification as described previously (Kuras and Struhl, 1999). Co precipitated DNA was used for PCR to amplify promoter fragments of ACR3 and YAP8 by using the following primers: ACR3 (5'-CTTTGCGTTCTGAAGAGAACTTTTCTCAGCAGGAGCTGCC-3') and ACR3 (5'-GGCAGCTGAATCTTCTCTTCAACAACCTCAGTGAAGACTGTCG-3'). The PCR products were separated in 2% agarose gel and visualized with ethidium bromide.

Cell Fractionation, Protein Extraction, and Western Analysis

Cells were grown in YPD, either untreated or exposed to 1.0 mM sodium arsenite for 3 h, nuclear extracts and cytoplasmic fractions were prepared and probed by Western analysis as described previously (Vongsamphanh et al., 2001).
Figure 1. Phenotypes of S. cerevisiae cells lacking AP-1 proteins and proteins involved in metalloid detoxification. (A) Metalloid sensitivity. Cells of W303-1A were grown in liquid medium, and 10-fold serial dilutions of the cultures were spotted on agar plates with or without metalloids. Growth was monitored after 3 d at 30°C. (B) Sensitivity of yap8Δ is limited to metalloids, whereas yap1Δ is sensitive to several oxidizing agents. Cells were cultivated as described above, and growth was monitored after 2–3 d at 25°C.

2001). Anti-myc antibodies (Sc-40; Santa Cruz Biotechnology, Santa Cruz, CA) were kindly provided by J.-Y. Masson (Laval University).

Fluorescence Microscopy

To analyze the distribution of GFP fusion proteins, transformants expressing the proteins of interest were grown in YNB medium lacking the appropriate amino acid to mid-log phase. To visualize DNA, 2 μg/ml 4’,6-diamino-2-phenylindole was added directly to the culture. Cells were washed twice with water or phosphate-buffered saline, and GFP signals were observed in living cells before and 10 min after exposure to 1 mM As(III), 10 mM Sb(III), or 0.5 mM H₂O₂ by using a Leica DM R fluorescence microscope.

RESULTS

Cells Lacking YAP1 and YAP8 Display Distinct Phenotypes

To investigate the role of the yeast AP-1–like proteins in metalloid tolerance, we first compared growth of the eight single yap mutants (yap1Δ to yap8Δ) to that of wild type (BY4741 background; EUROSCARF strain collection) in the presence of As(III), As(V), and Sb(III). The yap1Δ mutant was sensitive to As(III) and Sb(III) but grew as well as the wild type in the presence of As(V). The yap8Δ mutant displayed hypersensitivity to As(III) and As(V) and a slight sensitivity to Sb(III). Growth of the other yap mutants (yap2Δ to yap7Δ) was unaffected by the metalloids (our unpublished data). We then created single and double yap1Δ and yap8Δ mutants in the W303-1A background to test whether the phenotype is strain specific. The single W303-1A yap1Δ and yap8Δ mutants had the same distinct phenotypes as the corresponding BY4741 mutants (Figure 1A). The yap1Δ yap8Δ double mutant was clearly more sensitive to As(III) and As(V) than the single mutants, whereas the Sb(III) sensitivity of yap1Δ yap8Δ was only slightly higher than that of yap1Δ (Figure 1A). The data indicate a requirement of both Yap1p and Yap8p for As(III) and As(V) tolerance. In addition, Yap1p, and not Yap8p, is required for Sb(III) tolerance. Thus, the two transcriptional activators display both common and distinct metalloid specificity, indicating different biological functions and/or target genes.

Next compared growth and minimum inhibitory concentrations (MICs) of yap1Δ and yap8Δ mutants to those of yap7Δ and acr3Δ mutants lacking the transporters mediating metalloid detoxification (Figure 1A and Table 3). The MICs on As(III)-containing medium of yap1Δ (0.75 mM) and yap8Δ (0.20 mM) were very similar to those of ycf1Δ (0.75 mM) and acr3Δ (0.10 mM), respectively. Likewise, the MICs on As(III) of yap1Δ yap8Δ (0.07 mM) and acr3Δ ycf1Δ (0.05 mM) were similar. On As(V), yap8Δ and acr3Δ had identical MICs (both 1.0 mM). The ycf1Δ mutant was more strongly affected by Sb(III) (MIC of 0.25 mM) than yap1Δ (MIC of 2.0 mM). Collectively, these data indicate that Ycf1p plays a more important role in Sb(III) detoxification, whereas Acr3p is more active against As(III). Similarly, Yap8p is critical for As(III) and As(V) tolerance, whereas Yap1p protects cells against As(III) and Sb(III). To test whether Yap1p and Yap8p mediate tolerance through their known targets Ycf1p and Acr3p, respectively, we created yap1Δ ycf1Δ and yap8Δ acr3Δ double mutants and determined their MICs (Table 3). yap8Δ acr3Δ and acr3Δ cells had identical MICs under all conditions tested, suggesting that Yap8p mediates its effect via its target Acr3p. The yap1Δ ycf1Δ mutant was equally sensitive to As(V) and Sb(III) as yap1Δ, but showed a higher As(III) sensitivity than ycf1Δ. Hence, Yap1p might control expression of several additional genes required for As(III) detoxification (see further).

We expanded the growth analysis by including a variety of oxidative stress-generating agents in the growth medium (Figure 1B). Although yap1Δ was sensitive to a range of chemical oxidants, including diamide, menadione, methyl viologen (paraquat) and tert-butylhydroperoxide (t-BOOH), yap8Δ displayed parental resistance to these drugs. Moreover, the sensitivity of yap1Δ yap8Δ was identical to that of yap1Δ cells (Figure 1B). We conclude that unlike Yap1p, Yap8p is not involved in the general oxidative stress response.

Yap1p and Yap8p Have Different Subcellular Localizations

Yap1p has previously been shown to undergo cellular redistribution in response to oxidative stress (Kuge et al., 1997; Yan et al., 1998). We therefore tested whether metalloids could also alter Yap1p cellular localization. To do this, we introduced a centromeric plasmid containing Yap1p fused to GFP (Coleman et al., 1999) into yeast and followed the

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mM)</th>
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<tbody>
<tr>
<td></td>
<td>As(III)</td>
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<tr>
<td>Wild type</td>
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<tr>
<td>yap1Δ</td>
<td>0.75</td>
</tr>
<tr>
<td>yap8Δ</td>
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<tr>
<td>yap8Δ acr3Δ</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* MIC is the concentration at which no growth was observed on glucose medium. MIC values were determined on the basis of three independent experiments with identical results.

+ Sodium arsenate.

+ Sodium arsenate.

+ Potassium antimony tartrate.
distribution of the fusion protein in living cells (Figure 2A). In the absence of metalloids, GFP-Yap1p was evenly distributed throughout the cell. GFP-Yap1p showed a predominant nuclear signal (colocalization with 4′,6-diamino-2-phenylindole; our unpublished data) within 5 min of As(III) exposure (1.0 mM). The timing and the extent of GFP-Yap1p nuclear accumulation was similar in cells exposed to As(III) and to H$_2$O$_2$, which was used here as a positive control for oxidative stress (Figure 2A). Sb(III) exposure (10 mM) resulted in a somewhat weaker effect; GFP-Yap1p was present both in the cytoplasm and the nucleus and the nuclear signal was evident only after 10 min. This is somewhat surprising because Yap1p seems to play a more active role in detoxification of Sb(III) than of As(III) (Figure 1A). Nonetheless, the data clearly demonstrate that agents other than oxidants are able to trigger Yap1p activation and nuclear retention.

We also conducted parallel experiments to examine Yap8p cellular distribution in response to metalloid exposure. Because the YAP8 promoter did not produce sufficient amounts of the GFP-Yap8p fusion protein to allow detection when integrated into the genome, we placed GFP-Yap8p under the control of the galactose-inducible GAL1 promoter. To avoid massive overproduction of Yap8p that could lead to localization artifacts, cells were grown on raffinose and low galactose (0.5%). GFP-Yap8p was functionally active because it complemented the As(III) sensitivity of the yap8Δ mutant (Figure 6C). Direct fluorescence analysis revealed that GFP-Yap8p, in contrast to Yap1p, was already present in the nucleus of untreated cells (Figure 2A) and remained nuclear in cells exposed to As(III), Sb(III), or H$_2$O$_2$ (Figure 2A). We repeated the experiments by introducing a multicopy plasmid where the GFP-Yap8p was controlled by the endogenous YAP8 promoter into yeast. Also in this case, GFP-Yap8p was functional and showed a distinct nuclear localization under all conditions tested (Figure 2B; our unpublished data). To confirm nuclear residence of Yap8p, we created a genomic integration of a Myc-tag at the C-terminal end of the YAP8 gene. Nuclear and cytoplasmic extracts were prepared from untreated and As(III)-exposed cells expressing functional Yap8p-Myc and probed by Western blotting (Figure 2C). Yap8p-Myc was present in nuclear extracts both in untreated as well as As(III)-exposed cells, supporting the notion that Yap8p is a nuclear protein. We note, however, that a minor portion of Yap8p is present in the cytoplasm as observed both by fluorescence microscopy and by immunoblotting. Together, the data indicate that Yap1p and Yap8p have distinct cellular localizations under normal growth conditions and that they may be regulated in different ways to mediate metalloid stress-specific transcriptional activation.

**Yap1p and Yap8p Activate Expression of Distinct Subsets of Defense Genes**

We next followed the mRNA levels of various stress defense genes in metalloid-exposed cells. As(III) exposure strongly enhanced TRX2 (thioredoxin) and TRR1 (thioredoxin reductase) expression (5- and 2.5-fold, respectively), whereas induction of GSH1 (γ-glutamylcysteine synthetase) and GLR1 (glutathione reductase) was more moderate (both approximately twofold; Figure 3A). These induction levels are comparable with those observed upon cadmium (Dormer et al., 2000; Fauchon et al., 2002), or mercury (Westwater et al., 2002) exposure. Expression of other stress-responsive genes, including CTT1 (cytoplasmic catalase), HSP12 (putative LEA-protein), and GRE2 (similar to plant dihydroflavonol-4-reductases) was strongly stimulated by both As(III) and Sb(III), whereas only As(III) induced HSP104 (chaperone) expression (our unpublished data). Expression of the arsenic detoxification genes ACR2 and ACR3 was also enhanced, although these mRNAs generated weak signals that could not reliably be quantified (see below).

Consistent with the growth data, we found that Yap1p and Yap8p indeed control transcription of distinct subsets of defense genes. As(III)-induction of the oxidative stress-responsive genes TRX2, TRR1, GSH1, GLR1, and GRE2 was Yap1p dependent; their mRNA level was significantly reduced in the yap1Δ mutant compared with the wild type (Figure 3A). Expression of these genes was not significantly affected by YAP8 deletion. On the other hand, Yap8p was required for ACR2 and ACR3 expression; no expression of
Yap1p Is Hyperactivated in Metalloid-exposed Cells Lacking YAP8 or ACR3

We next examined control of YCF1 expression by using a YCF1 promoter-lacZ fusion construct (YCF1-lacZ) (Wemmie et al., 1994) (Table 4). YCF1-lacZ expression in wild-type cells was not affected by up to 20 h of metalloid exposure. Remarkably, YAP1 deletion did not affect YCF1-lacZ expression, whereas YAP8 deletion caused a nearly threefold increase in the presence of As(III) and As(V), but not Sb(III). This increase was Yap1p-dependent because YCF1-lacZ expression was reduced to basal level in yap1Δ yap8Δ cells. The reason for this observation might be that Yap8p impedes on Yap1p function, i.e., hinders the binding of Yap1p to the YCF1 promoter. Alternatively, Yap1p might be hyperactive in metalloid-exposed cells that lack Yap8p. To test the latter hypothesis, we monitored YCF1-lacZ expression in acr3Δ cells (Table 4). As(III) indeed stimulated YCF1-lacZ expression in the acr3Δ mutant (approximately twofold) albeit not as strongly as in yap8Δ (threefold). YCF1-lacZ expression was similar in yap8Δ acr3Δ and yap8Δ. Because yap8Δ and acr3Δ are equally sensitive to As(III), both possibilities might occur; hyperactivation of Yap1p in yap8Δ and acr3Δ mutants as well as an absence of Yap8p on the Yap1p-binding site of the YCF1 promoter in yap8Δ.

Similar observations were made using a GSH1 promoter-lacZ fusion construct (GSH1-lacZ) (Wu and Moye-Rowley, 1994) (Table 4). GSH1-lacZ expression in the wild type was moderately stimulated by As(III) (1.5-fold), As(V) (1.5-fold), and Sb(III) (twofold) in a Yap1p-dependent way (Table 4). In analogy with YCF1-lacZ, deletion of either YAP8 or ACR3 resulted in an approximately threefold higher GSH1-lacZ expression during metalloid exposure. Again, this stimulation was Yap1p-dependent because expression was reduced to the basal level when YAP1 was deleted in combination with either YAP8 or ACR3. In addition, the basal level of TRR1 mRNA was also increased in the yap8Δ mutant (Figure 3A). Hence, the consequences of an apparently hyperactive Yap1p can be seen on different promoters.

Activation of ACR2 and ACR3 Expression Requires a Putative AP-1 Binding Site

Although several studies have documented the promoter sequence recognized by Yap1p, the Yap8p recognition element has not previously been established (Toone and Jones, 1999; Toone et al., 2001). The promoter region between the divergently expressed ACR2 and ACR3 genes contains a putative AP-1 binding site with the sequence TTAATAA (Figure 4A). This sequence differs from the preferential Yap1p binding site TAATAAA on one position (underlined). The promoter of YAP8 itself contains such a TAATAAA sequence. To study the importance of this sequence for Yap8p-mediated As(III) tolerance, we deleted the TAATAAA sequence from the ACR3 promoter in plasmid pRW3, which contains the entire ACR3 gene, and transformed the resulting plasmid (mutACR3) into the acr3Δ strain. Cells containing the mutACR3 plasmid failed to grow in the presence of 0.5 mM As(III), whereas cells containing the ACR3 gene behind its native promoter grew as well as wild-type cells (Figure 4B). The fact that cells with the mutACR3 plasmid are unable to grow in the presence of As(III) is likely to be caused by a lack of ACR3 induction.

To confirm this notion, we deleted the TAATAAA sequence from the ACR2-lacZ and ACR3-lacZ constructs and measured β-galactosidase activity after metalloid exposure. Induction of ACR2-lacZ and ACR3-lacZ expression was completely lost in the promoter mutants (Figure 4C; our unpub-
lished data). Hence, induction of ACR2 and ACR3 expression by the metalloids is dependent on both Yap8p and the TTAATAA sequence.

Yap8p Interacts with the ACR3 Promoter In Vivo

The most straightforward explanation for the data mentioned above is that Yap8p controls ACR2 and ACR3 expression by direct binding to the promoter separating these genes (Figure 4A). To examine whether Yap8p is present on the ACR3 promoter in vivo, we performed chromatin immunoprecipitation assays by using two different strains: a strain expressing no FLAG-tagged Yap8p and a strain expressing FLAG-Yap8p. ChIP analysis revealed that FLAG-Yap8p was cross-linked with the ACR3 promoter (Figure 5).

A comparison of untreated and As(III)-exposed cells revealed that FLAG-Yap8p was present on the ACR3 promoter already in the absence of As(III)-stress and remained bound to this promoter in As(III)-exposed cells.

Because the Yap8p promoter also contains a TTAATAA sequence, we also investigated whether FLAG-Yap8p would bind to its own promoter. However, in contrast to the ACR3 promoter, FLAG-Yap8p was not associated with its own promoter, neither in the absence nor in the presence of As(III) (Figure 5). Hence, despite the fact that both promoters contain the TTAATAA sequence, Yap8p was only present on the ACR3 promoter, suggesting that other factors influence DNA-binding of Yap8p.

Identification of Yap1p and Yap8p Cysteines That Are Critical for Metalloid Tolerance

Yap8p has three cysteines in the n-CRD located at C121, C132, and C137. The position of C132 and C137 is conserved in both Yap1p and Yap8p (Figure 6A). Yap8p has one cysteine in a putative c-CRD (C274), which is at the same position as Yap1p-C629 (Figure 6A). We chose to create alanine substitutions in Yap8p at positions C132 and C274 by site-directed mutagenesis within the plasmid expressing GFP-Yap8p. These residues were chosen as they are conserved in the CRDs of both Yap1p and Yap8p as well as in several fungal AP-1-like proteins, including Schizosaccharomyces pombe Yap1 and Candida albicans Cap1 (Toone et al., 2001). Three mutants were created: GFP-Yap8p-C132A, GFP-Yap8p-C274A, and GFP-Yap8p-C132A C274A. The three Yap8p mutants had the same nuclear localization as the wild-type protein both in the absence (Figure 6B) and presence of metalloids (our unpublished data). Importantly, none of the mutants were able to complement the As(III) sensitivity of the yap8Δ mutant (Figure 6C). Moreover, neither of the single mutants GFP-Yap8p-C132A and GFP-Yap8p-C274A nor the double GFP-Yap8p-C132A C274A mutant was capable of inducing ACR3-lacZ expression upon metalloid exposure, whereas the native GFP-Yap8p protein (Yap8p) proved to be a strong activator of ACR3-lacZ expression in the presence of all metalloids tested (Figure 6D). It is noteworthy that the basal level of ACR3-lacZ was elevated due to higher amounts of GFP-Yap8p expression from the GAL1 promoter. From the above-mentioned data, we conclude that the cysteine residues at position C132 and C274 are essential for proper Yap8p function.

We expanded the analysis to include Yap1p mutants carrying alterations in the n- and c-CRDs; Yap1p-TAT (C598T C620A C629T), Yap1p-3Cys (C303T C310T C315T), and Yap1p-3Cys C620A (C303T C310T C315T C620A). These mutant proteins exhibit reduced nuclear residence in the presence of H2O2 or diamide and consequently a diminished expression of TRX2 (Kuge et al., 2001). We transformed the plasmids containing these GFP-tagged Yap1p mutants into yap1Δ cells and analyzed their capability to complement the yap1Δ phenotype. Importantly, the Yap1p-mutants complemented the As(III) sensitivity of yap1Δ to different degrees: Yap1p-3Cys produced wild-type growth, whereas Yap1p-TAT and Yap1p-3Cys C620A caused partial complementa-
As(III)-dependent activation of Yap1p, the mechanism by which As(III) activates the protein is clearly different from those involved in H₂O₂ and diamide-dependent activation.

**Figure 4.** ACR3 induction requires the TTAATAA promoter element. (A) Promoters of *ACR2*, *ACR3*, and *YAP8* contain putative AP-1 binding sites with the sequence TTAATAA. (B) Presence of the TTAATAA sequence in the *ACR3* promoter is required for Acr3p-mediated As(III) tolerance. Cells were grown in liquid medium and 10-fold serial dilutions of the cultures were spotted on agar plates with or without As(III). (C) Induction of *ACR3-lacZ* expression is dependent on the TTAATAA sequence. β-Galactosidase activity was measured as described in Figure 3.

**Figure 5.** Binding of FLAG-Yap8p to the *ACR3* promoter as detected by ChIP. PCR was performed on chromatin fragments isolated after immunoprecipitation (Anti-FLAG-IP) in cells expressing FLAG-Yap8p or cells without tagged protein (no tag) with primers that specifically amplify the *ACR3* and *YAP8* promoters. Cells were either untreated or exposed to 1 mM As(III) for 3 h. PCR was also performed on extracts that were not immunoprecipitated (input).

**DISCUSSION**

Our data provide important insight into the *S. cerevisiae* response to metalloid exposure and the involvement of the AP-1-like proteins Yap1p and Yap8p in tolerance acquisition. We show that yeast cells mount an oxidative stress response when challenged with metalloids; the mRNAs coding for proteins that maintain the cellular redox status (*TRX2*, *TRR1*), control cellular glutathione levels (*GSHello*, *GLR1*), or provide protection against oxidative damage (*CTT1*) were increased. Expression of other oxidative stress-responsive genes (*HSP12*, *GRE2*) was also stimulated. Indeed, As(III) has been shown to provoke increased ROS production in mammalian cells (Liu et al., 2001), and this may also be the case in yeast (our unpublished data). However, the mechanisms responsible for arsenic-induced ROS generation are poorly understood. Several possible mechanisms have been proposed, e.g., production of H₂O₂ as a result of As(III) oxidation or formation of hydroxyl radicals during the release of iron from ferritin triggered by arsenicals (Del Razo et al., 2001). It has also been postulated that arsenic trioxide induces apoptosis in acute promyelocytic leukemia cells by increasing the cellular H₂O₂ level possibly by inhibiting glutathione peroxidase (Jing et al., 1999). Hence, in addition to act directly upon the metalloids themselves, cells also increase their ability to deal with damages caused by oxidative stress.

Transcriptional activation of antioxidant genes was rapid; after exposure to metalloids, increased mRNA levels were detected within 15 min and peaked after 30–60 min. This is in contrast to the response of the arsenic detoxification genes *ACR2* and *ACR3* whose mRNAs were detected after 45–60 min and continued to increase up to 5 h. These results indicate that (expression of) these subsets of metalloid-responsive genes are controlled by different mechanisms and/or that their products may be involved at different stages during the adaptation process leading to tolerance. We did not find any evidence for enhanced *YCF1* expression during metalloid exposure, although its deletion clearly caused sensitivity. It is possible that an increase in the expression of *YCF1* requires prolonged exposure because two-
fold higher YCF1 expression was evident only after 24 h of exposure to cadmium (Li et al., 1997). Alternatively, the basal level of Ycf1p in the vacuolar membranes could be sufficient to mediate tolerance. Thus, conjugation of As(III) and Sb(III) to GSH may represent the rate-limiting step in tolerance acquisition. In that case, increased GSH synthesis might be sufficient to promote increased vacuolar sequestration of GSH-conjugated metalloids by the action of Ycf1p. A similar mechanism of arsenic tolerance was proposed in *Leishmania*, where increased synthesis of trypanothione, the major source of reduced thiols in trypanosomatidae, is required to produce resistance as a
result of formation and extrusion of metalloid–thiol complexes (Mukhopadhyay et al., 1996).

Collectively, it seems that the first line of defense during metalloid exposure is an increase in GSH1 synthesis coupled to Ycf1p activity as well as an oxidative stress response. The fact that gsh1Δ cells (when cultivated on minimal medium supplemented with cysteine to ensure growth) display sensitivity to As(III), As(V), and Sb(III) (our unpublished data) further corroborates this notion. When exposure persists, sensitivity to As(III), As(V), and Sb(III) (our unpublished data) is increased in GSH1 synthesis coupled to Ycf1p activity and transcription of Yap1p and transcriptional activation of its target genes, including TRX2 (thioredoxin). Mutations within the CRDs that affect Yap1p function under As(III) exposure (Yap1p-TAT and Yap1p-3Cys C620A) are indicated with an asterisk (*). Whether As(III) activates Yap1p through oxidative modification of critical cysteines or through another mechanism is presently unknown. (B) Yap8p is bound to the promoter of ACR3 (arsenic efflux protein) in an apparently inactive form in the absence of stress, whereas As(III) exposure activates Yap8p through an as yet unknown mechanism. Yap8p activation requires specific cysteine residues. Cysteine mutations (C132A or C274A) that affect Yap8p function are marked with an *.

Figure 7. Model for As(III)-activation of Yap1p and Yap8p. (A) As(III) triggers nuclear accumulation of Yap1p and transcriptional activation of its target genes, including TRX2 (thioredoxin). Mutations within the CRDs that affect Yap1p function under As(III) exposure (Yap1p-TAT and Yap1p-3Cys C620A) are indicated with an asterisk (*). Whether As(III) activates Yap1p through oxidative modification of critical cysteines or through another mechanism is presently unknown. (B) Yap8p is bound to the promoter of ACR3 (arsenic efflux protein) in an apparently inactive form in the absence of stress, whereas As(III) exposure activates Yap8p through an as yet unknown mechanism. Yap8p activation requires specific cysteine residues. Cysteine mutations (C132A or C274A) that affect Yap8p function are marked with an *.

Unlike Yap1p, Yap8p is a nuclear protein that does not exhibit stress-dependent changes in localization. Instead, ChIP analysis demonstrated that Yap8p is constitutively bound to the ACR3 promoter (Figure 7B). Interestingly, the Yap8p–Acr3p system seems to operate under prolonged As(III) exposure; induced ACR3 expression was only apparent after 45–60 min (mRNA) or after 20 h (β-galactosidase assay). Hence, although Yap1p controls the first-line defense, Yap8p is required for the cellular response during long-term exposure. Our data indicates that the control of Yap8p is neither exerted at the level of localization nor at the level of As(III)-stimulated binding to the ACR3 promoter. Hence, Yap8p activation may involve a novel mechanism.

Activation of Yap8p requires critical cysteine residues that are conserved in several fungal AP-1-like proteins. The Yap8p-C132A or Yap8p-C274A mutants were unable to induce ACR3 expression and, as a consequence, they could not produce As(III) tolerance. In contrast to yap1Δ, yap1Δ cells were not sensitive to chemical oxidants. Moreover, overexpression of Yap8p did not restore H2O2 tolerance of yap1Δ cells (our unpublished data). Although Yap8p activation by metalloid-induced oxidative modifications cannot be excluded solely based on these data, such an activation mechanism may seem less likely. Instead, it is conceivable that these Yap8p cysteines directly bind to As(III) inducing a conformational change such that the modified Yap8p can trigger gene expression.

Our data revealed that Yap8p-dependent activation of ACR2 and ACR3 expression requires a DNA sequence (TTATTAATAA) that is related to the DNA binding site of Yap1p (TTACTAA). Although these data suggest that Yap8p binds to the TTATTAATAA sequence, ChIP analysis did not show the presence of Yap8p on the YAP8 promoter, which also contains TTATTAATAA. It has recently been shown that mutations of nucleotides flanking the Yap1p and Yap2p DNA binding sites decreased expression of their target genes (Cohen et al., 2002). In analogy, the nucleotides flanking the TTATTAATAA of the two promoters are different; TTATTAATAATCAA in the ACR3 promoter and TTATTAATAATT in the YAP8 promoter, and this difference might affect DNA binding and expression of target genes. Although the exact sequence of the Yap8p–DNA binding–site remains to be determined, it is clear that the yeast AP-1-like proteins activate transcription through slightly different sequences. Yap1p to Yap4p preferentially interact with TTATCTAA, however, the strongest activation through this site is observed for Yap1p and much weaker for Yap2p and Yap3p (Fernandes et al., 1997). The promoter of the yeast major facilitator encoding Flr1 gene contains three functional but nonequivalent Yap responsive elements: YRE3 (TTACTAA), YRE2 (TGACTAA), and YRE1 (TTAGTCA). Yap1p binds to the YREs with different affinities (YRE3 > YRE2 > YRE1), and mutation of YRE3 caused the largest decrease in Flr1 activation (Nguyen et al., 2001). Finally, a recent microarray analysis has shown that expression of one cluster of genes that is specifically induced by Yap1p (but not by Yap2p) in response to H2O2 is signifi-
cantly increased in the absence of Yap2p (Cohen et al., 2002). Similarly, our data indicated higher expression of the Yap1p-dependent genes YCF1 and GSH1 in the absence of Yap8p. Hence, the yeast AP-1-like proteins may constitute a transcriptional network controlling expression of genes encoding protective functions under various adverse conditions. However, the precise nature of such a network remains to be elucidated.

We conclude that Yap1p and Yap8p mediate metalloid tolerance by activating transcription of distinct defense genes. However, the precise molecular mechanism(s) involved in metalloid-activation of Yap1p and Yap8p have yet to be revealed. Unveiling the tolerance mechanisms in yeast may prove of value for identifying similar mechanisms in other organisms and have important implications for the use of arsenic and antimony in medical therapy.

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