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

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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

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Table 1. *S. cerevisiae* strains used in this study

Strains:	Genotype	Source
W303-1A	<i>MATa ura3-1 leu2-3/112 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0</i>	Thomas and Rothstein (1989)
RW104	W303-1A <i>acr3Δ::loxP-kanMX-loxP</i>	Wysocki <i>et al.</i> (2001)
RW105	W303-1A <i>acr3Δ::loxP-kanMX-loxP ycf1Δ::loxP</i>	Wysocki <i>et al.</i> (2001)
RW117	W303-1A <i>yap8Δ::loxP</i>	This work
RW118	W303-1A <i>ycf1Δ::loxP</i>	Wysocki <i>et al.</i> (2001)
RW120	W303-1A <i>yap1Δ::loxP-kanMX-loxP yap8Δ::loxP</i>	This work
RW124	W303-1A <i>yap1Δ::loxP</i>	This work
RW127	W303-1A <i>yap1Δ::loxP-kanMX-loxP ycf1Δ::loxP</i>	This work
RW128	W303-1A <i>acr3Δ::loxP-kanMX-loxP yap1Δ::loxP</i>	This work
RW136	W303-1A <i>yap8Δ::loxP acr3Δ::loxP-kanMX-loxP</i>	This work
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf
YAP8-Myc	BY4741 YAP8-Myc <i>HIS3</i>	This work

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.*, 2000), whereas diamide activation involves the C-terminal cysteines C598, C620, and C629 (Kuge *et al.*, 2001). It has recently been shown that the glutathione peroxidase-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.

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.*, 2001; Toledano *et al.*, 2002). Overexpression of Yap2p confers cellular tolerance to cadmium and the cytotoxic drug 1,10-phenanthroline (Bossier *et al.*, 1993; Wu *et al.*, 1993), and it seems to control transcription of genes involved in protein folding and stabilization in an oxidative environment (Cohen *et al.*, 2002). Yap4p (Cin5p) and Yap6p (Hal7p) are nuclear proteins that confer resistance to the antitumor drug cisplatin when overexpressed (Furuchi *et al.*, 2001). Yap6p (Hal7p) and Yap7p (Hal6p) produce increased sodium and lithium tolerance when present in multiple copies (Mendizabal *et al.*, 1998). Previous studies indicated that Yap8p (Acr1p/Arr1p) is involved in arsenic tolerance, probably by controlling *ACR2* and *ACR3* expression (Bobrowicz *et al.*, 1997; Bobrowicz and Ulaszewski, 1998; Bouganim *et al.*, 2001). However, the molecular details of this regulation have not been investigated.

The goal of the present study was to explore the function of the *S. cerevisiae* AP-1-like proteins in metalloid tolerance. We show that Yap1p and Yap8p mediate arsenic and antimony tolerance by activating transcription of separate subsets of defense genes. We also provide evidence that metalloids activate these two proteins through distinct mechanisms.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Yeast strains used in this study are described in Table 1. All deletion mutants were constructed according to the method of Güldener *et al.* (1996) as described previously (Wysocki *et al.*, 2001). Yeast strains were grown on rich YPAD medium (1% yeast extract, 2% peptone, 2% glucose, 0.004% adenine sulfate) or on minimal YNB medium (0.67% yeast nitrogen base) supple-

mented with auxotrophic requirements and 2% glucose as a carbon source. Metalloid sensitivity assays were carried out as described previously (Wysocki *et al.*, 2001). Yeast transformations were performed by a modified lithium acetate method using PLATE mixture (Kaiser *et al.*, 1994). To create a genomic integration of the Myc-tag in the *YAP8* gene the PFA6a-Myc plasmid (kindly provided by J.-Y. Masson, Laval University, Québec, Canada) was used to amplify the fragment (YAP8-Myc) by polymerase chain reaction (PCR) by using the following primers: Yap8-Myc-F1 5'-T AG C C T C A A G C A T T T C A T T A A G G T C T T T T C G T C A A A T T A C G G A T C C C G G G T T A A T T A A 3' and Yap8-Myc-R1 5'-A T A A G A A A G A C A A T G T T G C G C T G T G C T T A C A G G A A G A A T A G A A T T C G A G C T C G T T T A A A C 3'. The resulting 2.1-kb fragment was integrated in frame at the penultimate codon into wild-type BY4741 strain, and proper integration was verified by PCR.

Plasmid Constructs

The plasmids used in this study are listed in Table 2. The *ACR3* promoter region from -352 to +34, where +1 represents the start of translation, was obtained by digesting plasmid pRW3 (Wysocki *et al.*, 1997) containing wild-type *ACR3*, with *KpnI* and ligating the promoter region into *KpnI*-digested pALTER-1 (Promega, Madison, WI) to generate the pALTER1-*ACR3* template for site-directed mutagenesis. Deletion of the TTAATAA sequence from the *ACR2-ACR3* intergenic region was achieved using the Altered Sites II in vitro mutagenesis system (Promega) and the mutagenic oligonucleotide MUT2 (5'-GCTCTTAATATCTTTTGTGATCAACITTAGCGCAACGCTCC-3'). The mutated fragment on plasmid pALTER1-mutACR3 was sequenced to confirm the desired mutation. *ACR3-lacZ* fusion plasmids were constructed by transferring an *EcoRI-BamHI* fragment from pALTER1-*ACR3* and pALTER1-mutACR3 into *EcoRI-BamHI*-cleaved pSEY102 (Emr *et al.*, 1986) to produce pEM19 and pEM20, respectively. The wild-type *ACR3* promoter from pRW3 was replaced with the *KpnI-KpnI* fragment from pALTER1-mutACR3. The resulting pRW33 plasmid contains the *ACR3* gene under the control of its endogenous promoter lacking the TTAATAA sequence. The *ACR2* promoter region from -323 to +6 was generated by PCR by using primers PE17 (5'-GCCTGCAGGGTTGCATCCTCGTTGGAGGT-3') and HACR2 (5'-CCCAAGCTTGATACCATACGCTTGCTGGATTG-3'), and the templates pALTER1-*ACR3* and pALTER1-mutACR3 to obtain wild-type and mutated forms of the *ACR2* promoter, respectively. The PCR products were cleaved with *HindIII-PstI* and cloned into YE357R (Myers *et al.*, 1986), producing plasmids *ACR2-lacZ* (pEM16) and mutACR2-*lacZ* (pEM18). The *ACR3* promoter fragment (from -354 to +14) was also cloned into *BamHI-PstI*-cleaved YE363 (Myers *et al.*, 1986) to produce the pRW11 plasmid with the *LEU2* marker. The GFP-*YAP1* fusion plasmid was kindly provided by S.W. Moye-Rowley (University of Iowa, Iowa City, IA) and has been described previously (Coleman *et al.*, 1999). Other GFP-*YAP1* plasmids expressing either wild-type Yap1p or the CRD mutants Yap1p-TAT, Yap1p-3Cys, and Yap1p-3Cys C620A were kindly provided by S. Kuge (Tohoku University, Sendai, Japan) and have been described previously (Kuge *et al.*, 2001).

To construct pGAL-GFP-*YAP8*, the *PIR1* gene was excised from plasmid pGAL-GFP-*PIR1* (Vongsamphanh *et al.*, 2001) with *BamHI-XbaI* and replaced by a PCR fragment containing *YAP8* by using wild-type genomic DNA as template and primers YAP8-F1-*BamHI* (5'-¹⁰GACCAAGAGGATCCCAAACCGCGTGGAAAGAAAAGCGGC^{+29-3'}) and YAP8-R2-*XbaI* (5'-¹⁰⁰⁶GGCGAAGGTA¹CTAGACGTAATAATCTTCACGCC^{+973-3'}) bearing the *BamHI* and *XbaI* sites (underlined), respectively. The *BamHI-XbaI*-cleaved PCR product was subcloned next to the green fluorescent protein (GFP) gene and placed under the control of the *GAL1* promoter to produce plasmid pGAL-GFP-*YAP8*. Cysteine to alanine mutations within Yap8p were created by the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA)

Table 2. Plasmids used in this study

Plasmid	Description	Source/Reference
pUG6	Vector containing the <i>loxP-kanMX-loxP</i> deletion cassette	Güldener <i>et al.</i> (1996)
YEp357R	2 μ vector with <i>lacZ</i> reporter gene, <i>URA3</i>	Myers <i>et al.</i> (1986)
YEp363	2 μ vector with <i>lacZ</i> reporter gene, <i>LEU2</i>	Myers <i>et al.</i> (1986)
pSEYC102	CEN vector with <i>lacZ</i> reporter gene, <i>URA3</i>	Emr <i>et al.</i> (1986)
GFP-Yap1p	pRS316 (CEN, <i>URA3</i>), Yap1p fused to GFP	Coleman <i>et al.</i> (1999)
pYES2	2 μ vector with <i>GAL1</i> promoter, <i>URA3</i>	Invitrogen
pGAL-GFP-Yap8p	GFP fused to N terminus of YAP8 in pYES2	This work
pEM16	ACR2- <i>lacZ</i> fusion in YEp357R	This work
pEM19	ACR3- <i>lacZ</i> fusion in pSEYC102	This work
pRW11	ACR3- <i>lacZ</i> fusion in YEp363	This work
pRW3	ACR3 in pFL39 (CEN, <i>TRP1</i>)	Wysocki <i>et al.</i> (1997)
pRW33	ACR3 behind its own promoter lacking the TTAATAA sequence (<i>mutACR3</i>) on pRW3	This work
pEM18	<i>mutACR2-lacZ</i> fusion lacking the TTAATAA sequence in YEp357R	This work
pEM20	<i>mutACR3-lacZ</i> fusion lacking the TTAATAA sequence in pSEYC102	This work
GFP-YAP8-C132A	C132A point mutation in pGAL-GFP-Yap8p (pYES2)	This work
GFP-YAP8-C274A	C274A point mutation in pGAL-GFP-Yap8p (pYES2)	This work
GFP-YAP8-C132A C274A	C132A C274A point mutations in pGAL-GFP-Yap8p (pYES2)	This work
pRS cp-GFP HA YAP1	GFP-fused Yap1p (wt) in pRS314 (CEN, <i>TRP1</i>)	Kuge <i>et al.</i> (1997)
pRS cp-GFP HA yap1 cm46A5	GFP-fused Yap1p-TAT (C598T C620A C629T) in pRS314	Kuge <i>et al.</i> (1997)
pRS cp-GFP HA yap1 3Cys	GFP-fused Yap1p-3Cys (C303T C310T C315T) in pRS314	Kuge <i>et al.</i> (2001)
pRS cp-GFP HA yap1 3Cys, C620A	GFP-fused Yap1p-3Cys C620A (C303T C310T C315T C620A) in pRS314	Kuge <i>et al.</i> (2001)
YEpl95-GFP-YAP8	GFP-YAP8 controlled by the endogenous YAP8 promoter in YEplac195 (2 μ , <i>URA3</i>)	This work
pJAW96	<i>YCF1-lacZ</i> fusion in pSEYC102	Wemmie <i>et al.</i> (1994)
pAW14	<i>GSH1-lacZ</i> fusion in pSEYC102	Wu and Moye-Rowley (1994)
pCM188	CEN vector, <i>URA3</i> , <i>tetO-CYC1</i> promoter	Gari <i>et al.</i> (1997)
pMit004	HIS ₆ -FLAG-tagged YAP8 in pCM188	This work

by using pGAL-GFP-YAP8 as template. The oligonucleotides used to create the C132A mutation were YAP8-C132A-A (5'-GCAACTGCTCTCAGTGGCT-GTTGGAAAGAATTGTACAGTGCCG-3') and YAP8-C132A-B (5'-CG-GCACTGTACAATTCTTTCCAACAGCCACTGAGAGCAGTTGC-3'), bearing the centrally located changes underlined. Similarly, the oligonucleotides YAP8-C274A-A (5'-CTTAAGAAAAGTGCTACGGCTTCTAATTTTGATATT-TTAATTAGCC-3') and YAP8-C274A-B (5'-GGCTAATTAATAATCAAAA-TTGAAGCCGTCAGACATTTTCTTAAG-3') were used to create the C274A mutation as well as the double mutation C132A C274A. The mutations in the resulting plasmids pYAP8-C132A, pYAP8-C274A, and pYAP8-C132A C274A were verified by DNA sequence analysis. The various GFP-YAP8 fusion proteins (wild-type and cysteine mutants) were also placed under the control of the endogenous YAP8 promoter. GFP-YAP8 was amplified by PCR by using the pGAL-GFP-YAP8 plasmids as templates and primers GFP-1 (5'-GCGGTACCATGAAAGGAGAAGAACTTTTC; *KpnI*-site underlined) and YAP8-R2-*XbaI* (see above). The YAP8 promoter was amplified using plasmid pA10 (Bobrowicz *et al.*, 1997) as template with the primers 5'-GGGGAATTC-CTCAAGCTTATTGTTCCAGC and 5'-GGGGGTACCTATCTTGGTCA-CTTATCTTCCG, bearing *EcoRI* and *KpnI* sites (underlined), respectively. The resulting PCR products were digested with *KpnI-XbaI* and *EcoRI-KpnI*, respectively, and cloned into *EcoRI-XbaI* digested YEplac195.

To construct HIS₆ and FLAG-tagged Yap8p, the YAP8 gene was amplified from genomic DNA by PCR by using the primers 5'-CATGCCATGGGTAT-GGCAAAACCGCGTGAAGA-3' and 5'-ACGCGTCGACTTATAATTTT-GACGAAAAGAC-3' bearing *NcoI* and *Sall* sites (underlined), respectively. The *NcoI-Sall* cleaved PCR product was cloned into plasmid pET30f (Ghislain *et al.*, 1996), and the resulting plasmid was sequenced to confirm in-frame ligation of YAP8 with HIS₆ and FLAG-tags. HIS₆-FLAG-YAP8 was in turn amplified by PCR by using the primers 5'-ATGCATCGATTAATACGACT-CACATAGGG (*Clal* site underlined) and 5'-GCTAGTTATTGCTCAGCGG, and the *Clal-NorI*-cleaved fragment was cloned into the yeast vector pCM188 (Gari *et al.*, 1997) to generate plasmid pMit004.

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 formaldehyde-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 quantified with a Molecular Dynamics PhosphorImager 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)], *yap8* Δ [0.05 mM As(III), As(V); 0.1 mM Sb(III)], *yap1* Δ [0.05 mM As(III), Sb(III); 0.1 mM As(V)], *yap1* Δ *yap8* Δ [0.05 mM As(III), As(V), Sb(III)]. For the last 2 h of incubation, the concentration of metalloids were 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 cross-linked 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 $\times$ complete 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 anti-mouse-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). Coprecipitated DNA was used for PCR to amplify promoter fragments of ACR3 and YAP8 by using the following primers: ACR3 (5'-CCTTCCTCTGATTTTCAATTAGGCC and 5'-GCGATTCACCATAT-TAACCTTAGAAGGTACCG) and YAP8 (5'-CCTCAAGCTTTATTGTTC-CAGC and 5'-TATCTTGGTCACTTATCTTCCG). 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.*,

1A

1B

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 metalloid salts. 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.

We next compared growth and minimum inhibitory concentrations (MICs) of *yap1Δ* and *yap8Δ* mutants to those of

Table 3. Sensitivity of yeast strains to arsenic and antimony salts

Strain	MIC ^a (mM)		
	As(III) ^b	As(V) ^c	Sb(III) ^d
Wild type	1.20	6.00	15.0
<i>yap1Δ</i>	0.75	5.00	2.00
<i>yap8Δ</i>	0.20	1.00	10.0
<i>yap1Δ yap8Δ</i>	0.07	0.75	1.00
<i>acr3Δ</i>	0.10	1.00	10.0
<i>ycf1Δ</i>	0.75	5.00	0.25
<i>acr3Δ ycf1Δ</i>	0.05	0.40	0.15
<i>yap1Δ ycf1Δ</i>	0.50	5.00	0.25
<i>yap8Δ acr3Δ</i>	0.10	1.00	10.0

^a 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.

^b Sodium arsenite.

^c Sodium arsenate.

^d Potassium antimonyl tartrate.

ycf1Δ 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 *ycf1Δ*, 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

Figure 2. Cellular localization of Yap1p and Yap8p. (A) Plasmids carrying GFP fusions of Yap1p and Yap8p were transformed into *yap1Δ* and *yap8Δ* cells, respectively. Transformants were cultivated in selective medium with either 2% glucose (GFP-Yap1p) or 2% raffinose + 0.5% galactose followed by induction with 2% galactose for 1–2 h (GFP-Yap8p). Living cells were analyzed by fluorescence microscope for Yap1p and Yap8p localization (GFP) before and 10 min after exposure to As(III) (1 mM), Sb(III) (10 mM), and H₂O₂ (0.5 mM). (B) Localization of GFP-Yap8p in *yap8Δ* cells transformed with a plasmid expressing the fusion protein from the endogenous *YAP8* promoter before and after 10 min of As(III) exposure. (C) Cells with a genomic copy of Yap8p fused to the Myc-tag were grown in YPD, either untreated or exposed to 1.0 mM As(III) for 3 h, and nuclear extracts and cytoplasmic fractions were prepared and probed by anti-myc antibodies. Cells lacking Yap8p-Myc was used as a control.

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 H₂O₂, 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 H₂O₂ (Figure 2A). We repeated the experiments by introducing a multi-copy 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), selenite (Pinson *et al.*, 2000), 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

Figure 3. Yap1p and Yap8p control As(III)-induced expression of different subsets of defense genes. (A) Northern blot analysis of total RNA extracted from wild-type, *yap1Δ*, *yap8Δ* and *yap1Δ yap8Δ* cells in the absence (lane 1) or presence of 1 mM As(III) for 60 min (lane 2) and 300 min (lane 3). The filter was hybridized to ³²P-labeled DNA fragments recognizing the indicated genes. (B) Metalloid-dependent induction of the *ACR3-lacZ* reporter in wild-type, *yap1Δ*, *yap8Δ* and *yap1Δ yap8Δ* cells. Cells exposed to metalloids were assayed for β -galactosidase activity as described in MATERIALS AND METHODS. The results are the average of three independent experiments. The error bars represent the SD.

these genes was detected in the *yap8Δ* mutant (see below). Finally, induction of *CTT1* and *HSP12* was somewhat reduced in *yap1Δ yap8Δ* cells, whereas *HSP104* expression was basically unaltered, suggesting that other transcription factors may be more important for the control of these genes (our unpublished data). Similar Yap1p- and Yap8p-dependent control of gene expression was obtained when cells were exposed to Sb(III) (our unpublished data).

To provide further evidence for the role of Yap8p in transcriptional control of *ACR2* and *ACR3*, we fused their promoters to the *lacZ* gene (*ACR2-lacZ* and *ACR3-lacZ*) and measured β -galactosidase activity in the same strains as above. *ACR3-lacZ* expression was induced 15-fold by As(III), 10-fold by As(V), and approximately fourfold by Sb(III) in wild-type cells transformed with the *ACR3-lacZ* fusion on a centromeric vector (PEM19) (Figure 3B). Deletion of *YAP8* resulted in a complete loss of *ACR3-lacZ* induction, whereas *YAP1* deletion did not significantly affect induction (Figure 3B). Similar results were also obtained using the *ACR2-lacZ* construct (our unpublished data). Collectively, the expression data clearly demonstrate a role of Yap1p in metalloid-dependent activation of various oxidative stress-responsive genes, whereas the main function of Yap8p seems to be linked to the control of *ACR2* and *ACR3* expression.

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 TTACTAA on one position (underlined). The promoter of *YAP8* itself contains such a TTAATAA sequence. To study the importance of this sequence for Acr3p-mediated As(III) tolerance, we deleted the TTAATAA 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 TTAATAA 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-

Table 4. Control of *YCF1-lacZ* and *GSH1-lacZ* expression in metalloid-exposed cells

Strain	<i>YCF1-lacZ</i> /pSEYC102 (U/OD ₆₀₀) ^a			
	No stress	As(III) ^b	As(V) ^b	Sb(III) ^b
Wild-type	5.4 ± 0.8	5.6 ± 0.3	5.2 ± 0.8	5.4 ± 0.2
<i>yap1Δ</i>	5.2 ± 0.1	4.1 ± 0.3	4.2 ± 0.3	3.6 ± 0.7
<i>yap8Δ</i>	6.1 ± 0.4	16.7 ± 0.9	13.9 ± 0.4	5.0 ± 0.6
<i>yap1Δ yap8Δ</i>	4.6 ± 0.5	4.8 ± 1.0	5.9 ± 0.4	3.5 ± 0.1
<i>acr3Δ</i>	5.1 ± 0.6	10.7 ± 0.4	8.6 ± 0.3	6.3 ± 1.1
<i>yap8Δ acr3Δ</i>	5.0 ± 0.15	15.9 ± 0.2	13.5 ± 0.45	5.9 ± 0.2
<i>yap1Δ acr3Δ</i>	4.0 ± 0.5	5.6 ± 1.0	6.1 ± 0.7	4.35 ± 0.4

Strain	<i>GSH1-lacZ</i> /pSEYC102 (U/OD ₆₀₀) ^a			
	No stress	As(III) ^b	As(V) ^b	Sb(III) ^b
WT	10.3 ± 0.6	15.0 ± 0.9	14.5 ± 1.3	18.3 ± 1.1
<i>yap1Δ</i>	7.0 ± 1.4	8.4 ± 1.5	9.4 ± 1.5	10.0 ± 1.1
<i>yap8Δ</i>	11.3 ± 0.7	32.8 ± 3.1	29.1 ± 2.1	20.2 ± 0.9
<i>yap1Δ yap8Δ</i>	7.6 ± 1.0	15.3 ± 1.7	13.5 ± 2.9	12.1 ± 1.5
<i>acr3Δ</i>	11.3 ± 1.2	32.9 ± 3.1	28.9 ± 3.4	21.0 ± 1.8
<i>yap8Δ acr3Δ</i>	11.9 ± 0.7	34.9 ± 2.9	29.5 ± 4.3	20.8 ± 1.9
<i>yap1Δ acr3Δ</i>	6.7 ± 0.7	13.3 ± 1.4	11.7 ± 1.9	10.7 ± 0.9

^a β -Galactosidase activities are the average of three independent experiments, including standard deviation.

^b *YCF1-lacZ* and *GSH1-lacZ* expression from the pSEYC102 centromeric plasmid after 20 h of metalloid preincubation (0.1 mM) followed by 2 h of induction with 1 mM respective metalloid.

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 *YAP8* 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* Pap1 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-

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.

tion. The latter two transformants were also sensitive to *t*-BOOH and diamide, whereas Yap1p-3Cys had a weaker phenotype (Figure 6E). All three alleles produced wild-type growth on Sb(III) medium (our unpublished data).

In agreement with the degree of complementation, Yap1p-3Cys showed strong nuclear retention upon As(III) exposure similar to that of wild-type Yap1p (Figure 6F). Yap1p-3Cys C620A showed an intermediate response; in the presence of As(III), the signal was obvious both in the nucleus and the cytoplasm, and the nuclear signal was more short-lived than that of wild-type Yap1p. In contrast, Yap1p-TAT showed a weak nuclear localization upon As(III) exposure, although most of the protein was in the cytoplasm. Moreover, no additional nuclear accumulation was detectable with Yap1p-TAT after prolonged exposure. It is possible that enough Yap1p-TAT enters the nucleus to provide partial As(III) tolerance (Figure 6E). We also noted that certain Yap1p mutants behaved differently under H₂O₂ and As(III) exposure (Figure 6F). Yap1p-TAT was mainly cytoplasmic in the presence of As(III), whereas H₂O₂ triggered a transient nuclear accumulation. Conversely, Yap1p-3Cys C620A was partially nuclear in As(III)-exposed cells, whereas H₂O₂ was unable to induce nuclear retention of the protein. Hence, despite the fact that the n- and c-CRDs seem to play a role in As(III)-dependent activation of Yap1p, the mechanism by

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).

which As(III) activates the protein is clearly different from those involved in H₂O₂ and diamide-dependent activation.

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 (*GSH1*, *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-

Figure 6. Functional analysis of Yap1p and Yap8p mutants bearing substitutions of conserved CRD cysteines. (A) Domain organization of Yap1p and Yap8p and position of conserved cysteine residues involved in redox regulation and metalloid tolerance. NES, nuclear export sequence. (B) Subcellular localization of Yap8p cysteine mutants. Plasmids carrying GFP fusions of Yap8p and the Yap8p-C132A, Yap8p-C274A, and Yap8p-C132A C274A mutants were transformed into *yap8Δ* cells, and the proteins were visualized by fluorescence microscopy. For simplicity, the GFP-Yap8p mutants are labeled in abbreviated form. (C) Yap8p cysteines C132 and C274 are both required for Yap8p function. *yap8Δ* cells were transformed with plasmids expressing either native GFP-Yap8p or the indicated GFP-Yap8p mutants. The *yap8Δ* mutant was also transformed with an empty control plasmid. Transformed cells were grown in liquid medium containing 2% raffinose and 0.5% galactose followed by induction with 2% galactose for 1–2 h. Serial 10-fold dilutions of the cultures were spotted on agar plates (with glucose as a carbon source) with or without As(III). (D) GFP-Yap8p cysteine mutants are unable to induce *ACR3-lacZ* expression. *yap8Δ* cells were transformed with empty control plasmid and with plasmids expressing either native GFP-Yap8p or the indicated GFP-Yap8p mutants. β -Galactosidase activity was measured as described in Figure 3. (E) Yap1p cysteines are important for As(III) and oxidative stress tolerance. *yap1Δ* cells were transformed with plasmids expressing either native GFP-Yap1p or the indicated GFP-Yap1p mutants. Wild-type and *yap1Δ* cells were also transformed with an empty control plasmid. Ten-fold serial dilutions of the cultures were spotted on agar plates and incubated at 30°C (As(III)) or 25°C (*t*-BOOH and diamide) for 2–3 d. For simplicity, the GFP-Yap1p mutants are labeled in abbreviated form. (F) Subcellular localization of Yap1p CRD mutants. Plasmids carrying GFP fusions of Yap1p and the indicated Yap1p mutants were transformed into *yap1Δ* cells, and the proteins were visualized before and 10 min after exposure to As(III) (1.0 mM) or H₂O₂ (0.5 mM) by fluorescence microscopy.

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

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 *.

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 GSH 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, expression of the detoxification system *ACR3* is stimulated to ensure continuous metalloid removal from the cytosol.

It is firmly established that various oxidants activate Yap1p and that the protein is required for oxidative stress tolerance as well as for resistance to heavy metals and cytotoxic agents (Toone *et al.*, 2001). Here, we show that metalloids also activate Yap1p (Figure 7A); the protein is detected in the nucleus within a few minutes of As(III) or Sb(III) exposure and transcription of Yap1p-dependent genes peaks at ~30–60 min. Moreover, this activation could be coupled with Yap1p-dependent expression of *GSH1*, *TRX2*, *TRR1*, *GLR1*, and *GRE2* under metalloid exposure; the induction of these genes is largely absent in *yap1Δ* cells. Hence, the metalloid sensitivity of *yap1Δ* can be attributed to, at least in part, a lack of transcriptional activation of oxidative stress defense genes.

The mechanism of Yap1p activation is clearly complex and may involve disulfide bond formation between distinct cysteines in an oxidant-specific manner. Yap1p mutants with modified cysteines in the c-CRD (Yap1p-TAT) or n-CRD in combination with C620A (Yap1p-3Cys C620A) displayed perturbed nuclear accumulation and were unable to

produce wild-type As(III) tolerance. In view of the fact that these Yap1p mutants responded differently to H₂O₂ and As(III), it is likely that these agents activate Yap1p through distinct mechanisms. Because *yap1Δ* cells are sensitive to a wide range of oxidants, it is tempting to speculate that Yap1p is regulated by metalloid-induced oxidative modifications of critical cysteines, either directly or via an auxiliary protein as is the case during H₂O₂ activation (Delaunay *et al.*, 2002). However, a more direct activation mechanism, e.g., binding of As(III) or Sb(III) to Yap1p, cannot be excluded at present.

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*, *yap8Δ* 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 (TTA-ATAA) that is related to the DNA binding site of Yap1p (TTACTAA). Although these data suggest that Yap8p binds to the TTAATAA sequence, ChIP analysis did not show the presence of Yap8p on the *YAP8* promoter, which also contains TTAATAA. 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 TTAATAA of the two promoters are different; TTGATTAATAATCAA in the *ACR3* promoter and TTCTTAATAAATT 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 TTACTAA, 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 (TGAATAA), 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, 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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REFERENCES

- Bobrowicz, P., and Ulaszewski, S. (1998). Arsenical-induced transcriptional activation of the yeast *Saccharomyces cerevisiae* *ACR2* and *ACR3* genes requires the presence of the *ACR1* gene product. *Cell Mol. Biol. Lett.* 3, 13–20.
- Bobrowicz, P., Wysocki, R., Owsianik, G., Goffeau, A., and Ulaszewski, S. (1997). Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. *Yeast* 13, 819–828.
- Bossier, P., Fernandes, L., Rocha, D., and Rodrigues-Pousada, C. (1993). Overexpression of *YAP2*, coding for a new yAP protein, and *YAP1* in *Saccharomyces cerevisiae* alleviates growth inhibition caused by 1,10-phenanthroline. *J. Biol. Chem.* 268, 23640–23645.
- Bouganin, N., David, J., Wysocki, R., and Ramotar, D. (2001). Yap1 overproduction restores arsenite resistance to the ABC-transporter deficient mutant *yef1* by activating *ACR3* expression. *Biochem. Cell Biol.* 79, 441–448.
- Cohen, B.A., Pilpel, Y., Mitra, R.D., and Church, G.M. (2002). Discrimination between paralogs using microarray analysis: application to the Yap1p and Yap2p transcriptional networks. *Mol. Biol. Cell* 13, 1608–1614.
- Coleman, S.T., Epping, E.A., Steggerda, S.M., and Moye-Rowley, W.S. (1999). Yap1p activates gene transcription in an oxidant-specific fashion. *Mol. Cell Biol.* 19, 8302–8313.
- Del Razo, L.M., Quintanilla-Vega, B., Brambila-Colombres, E., Calderon-Aranda, E.S., Manno, M., and Albores, A. (2001). Stress proteins induced by arsenic. *Toxicol. Appl. Pharmacol.* 177, 132–148.
- Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H₂O₂ sensing through oxidation of the Yap1 transcription factor. *EMBO J.* 19, 5157–5166.
- Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J., and Toledano, M.B. (2002). A thiol peroxidase is an H₂O₂ receptor and redox-transducer in gene activation. *Cell* 111, 471–481.
- Dormer, U.H., Westwater, J., McLaren, N.F., Kent, N.A., Mellor, J., and Jamieson, D.J. (2000). Cadmium-inducible expression of the yeast *GSH1* gene requires a functional sulfur-amino acid regulatory network. *J. Biol. Chem.* 275, 32611–32616.
- Emr, S.D., Vassarotti, A., Garrett, J., Geller, B.L., Takeda, M., and Douglas, M.G. (1986). The amino terminus of the yeast F₁-ATPase beta-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* 102, 523–533.
- Fauchon, M., Lagniel, G., Aude, J.C., Lombard, L., Soularue, P., Petat, C., Marguerie, G., Sentenac, A., Werner, M., and Labarre, J. (2002). Sulfur sparing in the yeast proteome in response to sulfur demand. *Mol. Cell* 9, 713–723.
- Fernandes, L., Rodrigues-Pousada, C., and Struhl, K. (1997). Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol. Cell Biol.* 17, 6982–6993.
- Furuchi, T., Ishikawa, H., Miura, N., Ishizuka, M., Kajiji, K., Kuge, S., and Naganuma, A. (2001). Two nuclear proteins, Cin5 and Ydr259c, confer resistance to cisplatin in *Saccharomyces cerevisiae*. *Mol. Pharmacol.* 59, 470–474.
- Gari, E., Piedrafit, L., Aldea, M., and Herrero, E. (1997). A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* 13, 837–848.
- Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* 11, 4241–4257.
- Ghislain, M., Dohmen, R.J., Levy, F., and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J.* 15, 4884–4899.
- Ghosh, M., Shen, J., and Rosen, B.P. (1999). Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 96, 5001–5006.
- Guarente, L. (1983). Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* 101, 181–191.
- Güldener, U., Heck, S., Fielder, T., Beinbauer, J., and Hegemann, J.H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24, 2519–2524.
- Jing, Y., Dai, J., Chalmers-Redman, R.M., Tatton, W.G., and Waxman, S. (1999). Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. *Blood* 94, 2102–2111.
- Kaiser, C., Michaelis, S., and Mitchel, A. (1994). *Methods in Yeast Genetics. A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Kuge, S., Arita, M., Murayama, A., Maeta, K., Izawa, S., Inoue, Y., and Nomoto, A. (2001). Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation. *Mol. Cell Biol.* 21, 6139–6150.
- Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J.* 16, 1710–1720.
- Kuras, L., and Struhl, K. (1999). Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme. *Nature* 399, 609–613.
- Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toledano, M.B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* 274, 16040–16046.
- Li, Z.S., Lu, Y.P., Zhen, R.G., Szczypka, M., Thiele, D.J., and Rea, P.A. (1997). A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc. Natl. Acad. Sci. USA* 94, 42–47.
- Liu, S.X., Athar, M., Lippai, I., Waldren, C., and Hei, T.K. (2001). Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc. Natl. Acad. Sci. USA* 98, 1643–1648.
- Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R., and de Larrinoa, I.F. (1998). Yeast putative transcription factors involved in salt tolerance. *FEBS Lett.* 425, 323–328.
- Mukhopadhyay, R., Dey, S., Xu, N., Gage, D., Lightbody, J., Ouellette, M., and Rosen, B.P. (1996). Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proc. Natl. Acad. Sci. USA* 93, 10383–10387.
- Mukhopadhyay, R., Shi, J., and Rosen, B.P. (2000). Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. *J. Biol. Chem.* 275, 21149–21157.
- Murray, H.W. (2001). Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob. Agents Chemother.* 45, 2185–2197.
- Myers, A.M., Tzagoloff, A., Kinney, D.M., and Lusty, C.J. (1986). Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* 45, 299–310.
- Nguyen, D.T., Alarco, A.M., and Raymond, M. (2001). Multiple Yap1p-binding sites mediate induction of the yeast major facilitator *FLR1* gene in response to drugs, oxidants, and alkylating agents. *J. Biol. Chem.* 276, 1138–1145.
- Pinson, B., Sagot, I., and Daignan-Fornier, B. (2000). Identification of genes affecting selenite toxicity and resistance in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 36, 679–687.
- Tamás, M.J., and Wysocki, R. (2001). Mechanisms involved in metalloid transport and tolerance acquisition. *Curr. Genet.* 40, 2–12.

- Thomas, B.J., and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell* 56, 619–630.
- Toledano, M.B., Delaunay, A., Biteau, B., Spector, D., and Azevedo, D. (2002). Oxidative stress responses in yeast. In: *Yeast Stress Responses*, vol. 1, ed. S. Hohmann and W.H. Mager, Heidelberg: Springer, 241–303.
- Toone, W.M., and Jones, N. (1999). AP-1 transcription factors in yeast. *Curr. Opin. Genet. Dev.* 9, 55–61.
- Toone, W.M., Morgan, B.A., and Jones, N. (2001). Redox control of AP-1-like factors in yeast and beyond. *Oncogene* 20, 2336–2346.
- Waxman, S., and Anderson, K.C. (2001). History of the development of arsenic derivatives in cancer therapy. *Oncologist* 6 (suppl 2), 3–10.
- Wemmie, J.A., Szczypka, M.S., Thiele, D.J., and Moye-Rowley, W.S. (1994). Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. *J. Biol. Chem.* 269, 32592–32597.
- Westwater, J., McLaren, N.F., Dormer, U.H., and Jamieson, D.J. (2002). The adaptive response of *Saccharomyces cerevisiae* to mercury exposure. *Yeast* 19, 233–239.
- Vongsamphanh, R., Fortier, P.K., and Ramotar, D. (2001). Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability. *Mol. Cell. Biol.* 21, 1647–1655.
- Wu, A., Wemmie, J.A., Edgington, N.P., Goebel, M., Guevara, J.L., and Moye-Rowley, W.S. (1993). Yeast bZip proteins mediate pleiotropic drug and metal resistance. *J. Biol. Chem.* 268, 18850–18858.
- Wu, A.L., and Moye-Rowley, W.S. (1994). GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. *Mol. Cell. Biol.* 14, 5832–5839.
- Wysocki, R., Bobrowicz, P., and Ulaszewski, S. (1997). The *Saccharomyces cerevisiae* ACR3 gene encodes a putative membrane protein involved in arsenite transport. *J. Biol. Chem.* 272, 30061–30066.
- Wysocki, R., Chéry, C.C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J.M., and Tamás, M.J. (2001). The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 40, 1391–1401.
- Yan, C., Lee, L.H., and Davis, L.I. (1998). Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *EMBO J.* 17, 7416–7429.