The Saccharomyces cerevisiae AP-1 Protein Discriminates between Oxidative Stress Elicited by the Oxidants H$_2$O$_2$ and Diamide

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The Saccharomyces cerevisiae AP-1 protein (yAP-1) is a key mediator of oxidative stress tolerance. Transcriptional activation by yAP-1 has been shown to be inducible by exposure of cells to H$_2$O$_2$ and diamide, among other oxidative stress eliciting compounds. Here we define the segments of the yAP-1 protein that are required to respond to this environmental challenge. Western blotting analyses indicated that levels of yAP-1 do not change during oxidative stress. Deletion mutagenesis and gene fusion experiments indicate that two different segments of yAP-1 are required for oxidative stress inducibility. These two domains function differentially depending on the type of oxidant used to generate oxidative stress. Three repeated cysteine-serine-glutamate sequences located in the carboxyl terminus are required for normal regulation of yAP-1 function during oxidative stress. Replacement of these cysteine-serine-glutamate repeats by alanine residues does not similarly affect H$_2$O$_2$ and diamide regulation of yAP-1 function. While yAP-1 transactivation is enhanced by exposure to either H$_2$O$_2$ or diamide, the protein responds to the oxidative stress produced by these compounds in nonidentical ways.

Oxidative stress is a challenge faced by all cells that grow in an aerobic environment. Cellular damage resulting from oxidative stress has been implicated in a wide variety of pathological conditions including Down’s Syndrome (1), familial amyotrophic lateral sclerosis (2), and cancer (3) as well as normal cellular processes like aging (4) and apoptosis (5). Not surprisingly then, cells possess a robust ability to detoxify the redox environment (6–8). These regulatory proteins are likely to act to link oxidative stress to a downstream response to this challenge.

The yeast Saccharomyces cerevisiae serves as an invaluable model system for fundamental eukaryotic processes such as oxidative stress tolerance. As first shown by Schnell et al. (9), the YAP1 locus is a key determinant of oxidative stress tolerance. YAP1 encodes a basic region-leucine zipper (bZip) transcription factor of 650 amino acids that is a positive regulator of gene expression (9–12). Domain mapping experiments indicate the presence of two separable transactivation domains in the protein, located between amino acids 220–379 and 430–537 (13). We have found that transcriptional control of GSH1 and production of normal levels of glutathione require the action of yAP-1 (14). Kuge and Jones (15) demonstrated that both an artificial yAP-1-responsive reporter and TRX2 gene expression were inducible by a variety of oxidative stress agents in a yAP-1-dependent fashion. The induction of yAP-1-dependent transactivation correlated with an increase in yAP-1 DNA binding activity.

In this work, we have explored the yAP-1 protein sequences required for oxidative stress induction of yAP-1 transactivation. Through the use of deletion mutagenesis and lexA fusion proteins, two different regions of yAP-1 have been identified that mediate the response of this protein to H$_2$O$_2$ and diamide-induced oxidative stress. Intriguingly, while both of these two regions affect the response of yAP-1 to H$_2$O$_2$ and diamide, the consequences of mutating either region varies with the type of oxidant used to generate stress. These data strongly suggest that oxidative stress conditions generated by H$_2$O$_2$ and diamide are differentially sensed by yAP-1.

MATERIALS AND METHODS

Yeast Methods—The yeast strains used in this study are: SEY6210 (MATa, leu2-3,112, ura3-52, his3–Δ200, trp1–Δ901, lys2–801, suc2–9, Mel–), SM12 (MATa, leu2-3,112, ura3-52, his3–Δ200, trp1–Δ901, lys2–801, suc2–9, Mel–, yap1–Δ1::HIS3, ARE-TRP5-lacZ), SM13 (MATa, leu2-3,112, ura3-52, his3–Δ200, trp1–Δ901, lys2–801, suc2–9, Mel–, yap1–Δ32::hisG), and YSM13 (MATa, leu2-3,112, ura3-52, his3–Δ200, trp1–Δ901, lys2–801, suc2–9, Mel–, lexA-3GAL1-lacZ). Yeast cells were grown either in rich, non-selective medium (YPD), minimal medium (SD) with required supplements or minimal medium supplemented with casamino acids (16). Transformation was carried out using the LiOAc technique of Ito et al. (17). β-Galactosidase assays were performed on permeabilized cells as described previously (18). SM13 was constructed from SEY6210 by introducing the yap1–Δ32::hisG allele. This was accomplished by isolation of a KpnI/SacI fragment from pS107 and transformation of this fragment into SEY6210 with selection for URA3. URA3 transformants were analyzed by Southern blotting to confirm the presence of the yap1–Δ32::hisG allele. The URA3 gene was cured via treatment with 5-fluoroorotic acid (19). A lexA-3GAL1-lacZ reporter gene (pSH18–343G) was integrated into the URA3 gene in SEY6210 by ApaI cleavage as described1 to form the lexA-responsive reporter strain YSM13.

Plasmids—The construction of the carboxyl-terminal and internal deletion series of YAP1 derivatives has been described before (13). The yap1–Δ32::hisG allele was generated by insertion of a BamHI/SacI fragment, containing hisG::URA3::hisG, from the plasmid pNY51, into BamHI/SacI-cleaved pJAW4 (13). The resulting construct, pSM107, contains a yap1 allele lacking amino acids 64–469. This new yap1 allele was constructed to replace the previous yap1–Δ1::HIS3 allele (20). This

1 S. Hanes, personal communication.
was done to prevent introduction of the yeast promoters associated with each end of the BamHI fragment carrying the HIS3 gene (21) which could lead to production of truncated but still immunoreactive yAP-1 protein species.

Construction of the chimeric yAP-1-PV16 fusion protein was accomplished via insertion of the VP16 transactivation domain into pSM1 (13) that had been treated with SalI, end repaired with the Klenow fragment, and then digested with HindIII. The VP16 transactivation domain was obtained from the pCR3 plasmid (22) as a Klenow-repaired BglII/HindIII segment. This plasmid was named pSM1S7 and expressed the 219 NH2-terminal residues of yAP-1 fused to the VP16 transactivation domain. To express yAP-1 chimeras lacking the YAP1 promoter and NH2 terminus, a plasmid was constructed to provide a yeast promoter and convenient cloning sites. This expression plasmid, pAK1, was generated by inserting an ADH1 promoter segment as a HindIII/SalI fragment from yAP-1 into pSM1/SalI-cut pEG202, a lexA expression plasmid (24). The lexA-VP16 fusion gene was then transferred into pAK1 as a HindIII/SalI fragment. Similarly, the lexA-yAP-1 fusion was constructed by inserting a HpaI-BamHI linker/SalI fragment from yAP-1 into pSM1/SalI-cut pEG202. This lexA-yAP-1 fusion protein consisted of the lexA DNA-binding domain fused to residues 156–650 of yAP-1. This fusion gene was then transferred into pAK2 as a HindIII fragment to generate pAK2.

The alanine scanning mutations (25) were generated via polymerase chain reaction using a two-step procedure as described (26). The mutagenic primers were: Site 1, GCC TCT TTA CTA AGG GCC GGC GCA ATT TGG GAT AGA ATA ACA, Site 2, GAT GTC GAT GGT TTA GCC GGC GCA CTA ATG GCA AGA GCA AAA GCC GGG GCA AGA GGT GTC AAT. Note that each alanine scanning mutation introduces a new SacII restriction site into the yAP1 coding sequence. All mutant forms of yAP1 were sequenced in their entirety to avoid introduction of spurious mutations along with the desired alanine scanning lesion. Each alanine scanning mutation was cloned as a YAP1/HindIII fragment into pJAW15 (13), replacing a wild-type version of this fragment. Presence of the desired mutation(s) was confirmed by cleavage with SacII.

Production of Anti-yAP-1 Rabbit Polyclonal Serum—Production of yAP-1 was carried out in bacteria using a histidine-tagged form of yAP-1. A BamHI/SalI fragment encoding yAP-1 residues 63–650 was inserted into the plasmid pET28b+ (Novagen). The resulting fusion protein contained six histidine residues fused in-frame with the large yAP1 carboxyl-terminal fragment. This fusion was then transferred to the yeast mid-region containing pOTSoN12 (27) as an Asp7/SalI fragment. This new chimera (pJAW140) contained the polyhistidine-yAP1 fusion gene under control of the heat-inducible αl promoter. pJAW140 was transformed into an Escherichia coli strain (AR68) containing the lacZ reporter under control of the heat-inducible αl promoter. Protein extracts were prepared as described (10) from heat-induced AR68 transformants carrying pJAW140. Purification of the polyhistidine-yAP1 fusion protein was accomplished through nickel chelate chromatography as described by the manufacturer (Novagen). Purified fusion protein was then used to immunize New Zealand White rabbits using standard techniques (28). The crude antiserum was then directly used for Western blot analysis.

Western Blotting Analysis—For Western blotting, cells were grown in 50 ml of minimal media to an A600 of 0.6; drug was added, and cells were incubated for an additional 1.5 h. Diamide or hydrogen peroxide was added to give final concentrations of 1.5 or 1.0 mM, respectively. Cells were harvested, washed, and broken by glass bead lysis in buffer containing 300 mM sorbitol, 100 mM NaCl, 5 mM MgCl2, 10 mM Tris, pH 7.4, and protease inhibitors. Cell lysate was cleared and the Bradford protein assay was used to determine the protein concentration of the supernatant. 100 µg of protein of each sample was run on a 5–15% polyacrylamide gradient gel. Proteins were transferred to nitrocellulose, blocked with 2.5% nonfat dry milk in phosphate-buffered saline, and probed with the anti-yAP1 polyclonal antisera or a mouse monoclonal antibody directed against carboxypeptidase Y (YAP1) (Y2) with the ECL kit (Pierce) were used to visualize immunoreactive protein.

RESULTS

Oxidative Stress Stimulation of Wild-type yAP-1—As a reporter system for yAP-1-dependent transactivation, we employed the yeast strain SM12. This strain contains a deletion allele of the chromosomal YAP1 locus and an integrated copy of a YAP1-dependent gene fusion (ARE-TRP5-lacZ). A low-copy vector plasmid either carrying or lacking the YAP1 structural gene was introduced into the SM12 background. Appropriate transformants were then assayed for yAP1-dependent transactivation of the ARE-TRP5-lacZ fusion gene.

Expression of SM12 transformants carrying the wild-type YAP1 gene to the oxidative stress agents diamide, H2O2, or diethylmaleate led to a strong induction in ARE-dependent β-galactosidase activity (Fig. 1). The presence of a carboxy-terminal deletion mutant of yAP1 that has been previously shown to be transcriptionally inactive (13) abolished oxidative stress response.
stress-inducible lacZ expression. These results establish that the β-galactosidase activity produced by the ARE-TRP5-lacZ fusion gene faithfully reproduces the previously described elevation of yAP-1-dependent transcription elicited by oxidative stress (15) and that the COOH terminus of the protein is required for this response.

Western blot analysis was next carried out to directly examine yAP-1 protein levels during oxidative stress. Protein extracts were prepared from control and oxidatively-stressed cells, electrophoresed on SDS-PAGE,2 and transferred to nitrocellulose. The location of immunoreactive yAP-1 was detected through use of the rabbit polyclonal anti-yAP-1 antisera. In response to oxidative stress elicited by diamide, H2O2, or diethylmaleate, steady-state levels of yAP-1 did not change (Fig. 1). Probing the same membrane with a monoclonal antibody against carboxypeptidase Y demonstrated that approximately equal amounts of protein were loaded in each sample. This observation provides definitive evidence that the elevation in yAP-1-dependent transactivation does not involve an increase in the level of yAP-1 protein. A previous examination of this issue employed Northern blotting as the assay for yAP-1 synthesis and did not directly examine protein levels of the factor (15). Additionally, pulse-chase experiments indicate that the stability of the yAP-1 protein did not change in response to oxidative stress.3 These data establish that oxidative stress modulation of yAP-1 function occurs through post-translational modification of the factor.

A lexA-YAP1 Gene Fusion Is Responsive to Oxidative Stress—To identify the regions of yAP-1 that are required for oxidative stress induction of yAP-1 transactivation, we employed a set of carboxyl-terminal truncation mutations of this protein (13). This analysis was not informative as even a mutant form of YAP1 consisting of amino acids 1–627 was unable to significantly activate ARE-TRP5-lacZ expression, failed to complement the H2O2 or diamide hypersensitivity of a Δyap1 strain, and was not detectable by Western blotting (data not shown). We concluded from this analysis that the carboxyl terminus of yAP-1 was dispensable for normal steady-state levels of the protein and possibly for function. To analyze and compare the role of the yAP-1 amino- and carboxyl-terminal segments in oxidative stress induction, we prepared chimeras between these yAP-1 protein domains and appropriate heterologous domains from other transcription factors. These chimeras were then tested for their effect on oxidative stress induction of appropriate reporter genes.

A carboxyl-terminal fragment of yAP-1, extending from amino acid 156 to 650, was fused to the bacterial lexA protein (29). This lexA-yAP1 chimera was then expressed in an S. cerevisiae strain carrying a GAL1-lacZ fusion with multiple lexA operator (lexAop) sites in place of the normal GAL1 UAS (29, 30). As a control for the specific oxidative stress inducibility of the lexA-yAP1 fusion protein, a lexA-VP16 fusion protein was also expressed in these cells. β-Galactosidase expression from the lexAop-GAL1-lacZ fusion gene was then assayed under non-stressed and H2O2- or diamide-stressed conditions (Table 1). The lexA-yAP1 fusion protein was able to respond to diamide induction but not to exposure to H2O2. β-Galactosidase activity produced in response to the presence of the lexA-VP16 fusion protein was not responsive to either oxidative stress agent. Western blotting experiments using the anti-yAP-1 antisera indicated that the lexA-yAP1 fusion protein was produced at equivalent levels under non-stressed and stressed conditions (data not shown). This result supports the view that

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2 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
3 S. Steggerda, unpublished data.
The level of H$_2$O$_2$-dependent β-galactosidase activity produced by this mutant protein represented 6% of wild-type H$_2$O$_2$- dependent expression and only 22% of the activity this same mutant elicited in the presence of diamide. A yAP-1 derivative lacking amino acids between 220 and 469 was not able to respond to H$_2$O$_2$-produced oxidative stress. These data strongly suggest that the 220–335 region serves to inhibit the ability of yAP-1 to transactivate in response to diamide but is required for normal transactivation during H$_2$O$_2$-elicited stress. This assertion is supported by the observation that none of this group of three internal deletion mutations was able to normally complement the H$_2$O$_2$ hypersensitivity of a $\Delta$ haploid mutant strain (see below).

Each internal deletion mutant allele was also tested for the ability to complement the diamide and H$_2$O$_2$ hypersensitivity of a strain lacking the YAP1 locus (Fig. 3). As predicted from the relative degree of activation of the ARE-TRP5-lacZ fusion gene, the yAP-1Δ220–335 protein elevated diamide tolerance relative to the wild-type factor. In agreement with its defect in H$_2$O$_2$ induction of reporter gene expression, the yAP-1Δ220–335 protein was not able to normally complement the H$_2$O$_2$ hypersensitivity caused by lack of the YAP1 gene. The other two internal deletion mutant proteins lacking the 220–335 region (yAP-1Δ220–430 and yAP-1Δ220–469) were both able to grow at least weakly on diamide plates but were unable to complement the H$_2$O$_2$ hypersensitive phenotype of the $\Delta$ yap1 strain. The remaining internal deletion mutants were able to correct both the diamide and H$_2$O$_2$ hypersensitive phenotypes of the $\Delta$ yap1 strain.

Western blotting analysis was also carried out on each internal deletion mutant protein to assess the relative steady-state levels of each factor (Fig. 4). Protein extracts were prepared from selected $\Delta$ yap1 transformants expressing each mutant factor under normal and diamide- or H$_2$O$_2$-stressed conditions. Equal amounts of protein were resolved on SDS-PAGE and the relative levels of immunoreactive yAP-1 assessed by probing with the anti-yAP-1 antiserum.

All internal deletion mutant proteins were produced at comparable levels and migrated at the expected molecular mass, irrespective of the presence of diamide as a stress agent (Fig. 4). The same analysis was performed for selected mutant proteins using H$_2$O$_2$ as the stress agent. The levels of the mutant proteins did not change in response to the different oxidative stress agents. We conclude that the observed differences in transactivation and inducibility of the various yAP-1 derivatives are due to alterations in the function of each mutant protein rather than the level of production.

### Oxidative Stress Response of yAP-1

A *Repeated Amino Acid Triplet Important in Activation of yAP-1*.—While the above analysis of yAP-1 localized a major oxidative stress responsive segment of the polypeptide, no information is available on specific residues that might contribute to regulation of yAP-1. In other oxidative stress-responsive factors, including c-Jun (31), SoxR (32), and OxyR (33), cysteine residues have been implicated in oxidative stress modulation of function. The carboxyl-terminal 52 amino acids of yAP-1 possesses three cysteine residues, present as cysteine-serine-glutamic acid (CSE) triplet repeats. The three CSE repeats begin with cysteine at positions 598, 620, and 629 and are referred to as sites 1, 2, and 3, respectively. To assess the role of each CSE repeat in regulation of yAP-1, we prepared alanine scanning mutations in each of these CSE repeats and analyzed the function of the resulting yAP-1 derivative. Three single CSE and three double CSE alanine scanning mutations were generated.

The site 1 and site 2 mutant proteins were both defective in their ability to be regulated by oxidative stress (Table III). The site 1 mutant had a pronounced defect in the ability to activate the ARE-TRP5-lacZ fusion under normal conditions but was still able to be induced by diamide or H$_2$O$_2$ exposure, although

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**Table II**

| Transcription (factor present) | ARE-TRP5-lacZ Expression ($\mu$D$_{\text{max}}$) |
|-------------------------------|----------------------------------|
| YAP1                          | 45                              |
| yAP1-Δ219                     | $<0.3$                          |
| yAP1-Δ219-VP16                | 38 ± 7                          |
| Pdr3p-VP16                   | $<0.3$                          |
| Vector                       | $<0.3$                          |

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*SM12 (relevant genotype: yap1Δ1::HIS3, ARE-TRP5-lacZ, ura3Δ52) cells were transformed with URA3-containing plasmids containing the indicated fusion construct. Appropriate transformants were grown in SD medium plus necessary supplements and either not treated (no stress) or subjected to oxidative stress with the addition of diamide (diamide) or hydrogen peroxide (H$_2$O$_2$) as described above. ARE-dependent β-galactosidase activities are presented. Pdr3p-VP16 served as an unrelated DNA-binding domain-VP16 gene fusion (44).*

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*Each type of transcription factor listed below was introduced on a plasmid. All YAP1 derivatives were carried on a low-copy plasmid while the PDR3-VP16 gene was cloned into the 2-$\mu$m vector pRS426. Vector refers to the presence of the low-copy vector pRS316 lacking any yeast transcription factor gene.*
the induction by H$_2$O$_2$ was reduced to only 14% of wild-type. Loss of the CSE repeat at site 2 produced a factor that had higher than normal transactivation function under non-stressed conditions but was not significantly inducible upon oxidative stress. The site 3 mutant protein had a dramatic defect in regulation and produced 1325 units/1.4 mM H$_2$O$_2$. The loss of the site 3 CSE led to a mutant yAP-1 gene, while vector represents a transformant containing the vector plasmid lacking YAP1 sequences.

Each alanine scanning mutant protein was also assessed for the ability to confer oxidative stress resistance to a Δyap1 strain when introduced on a low-copy plasmid vector. As expected from the behavior of the ARE-TRP5-lacZ fusion gene, all mutants lacking the site 3 CSE element exhibited a striking elevation in diamide tolerance (Fig. 5). The site 3 mutants were all more resistant to diamide than the wild-type protein. Mutant yAP-1 derivatives lacking only site 1, site 2, or both of these sites produced diamide resistance that was similar to that of the wild-type factor. Both the site 1 and site 3 single mutant factors were defective in their ability to restore H$_2$O$_2$ resistance to a cell lacking a chromosomal YAP1 locus while a strain was next analyzed (Fig. 6). These altered yAP-1 derivatives were capable of high-level transactivation of the ARE-TRP5-lacZ in the presence of H$_2$O$_2$. We believe this discrepancy is caused by the high levels of β-galactosidase elicited in the absence of oxidative stress and the marked stability of this enzyme in yeast (34). This and other possible reasons for the differential H$_2$O$_2$ behavior of the site 3 mutant protein are discussed below. All of the three double mutant proteins were defective in their ability to complement H$_2$O$_2$ hypersensitivity. The steady-state level of each single CSE mutant protein was next analyzed (Fig. 7). These altered yAP-1 derivatives were produced under control or diamide or H$_2$O$_2$-stressed conditions and subjected to Western blot analysis as described above. Alteration of sites 1 and 2, alone or in combination, led to the loss of the site 3 CSE element produced markedly reduced levels of immunoreactive yAP-1. The steady-state level of each double mutant protein was also assessed (data not shown). Alteration of sites 1 and 2, alone or in combination, led to the loss of the site 3 CSE element produced markedly reduced levels of immunoreactive yAP-1.
The transcriptional response to oxidative stress can be broken down into two phases: early or primary responses and later or secondary responses. Primary response genes are likely to act to provide rapidly acting buffers of oxidants. The function of NF-κB in the response of S. cerevisiae to oxidative stress is most consistent with this factor playing a role as a primary response regulator. Transactivation by yAP-1 is strongly induced over a short time period and this induction does not require an increase in translation of yAP-1. Additionally, two known yAP-1 target genes, OSH1 (14) and TRX2 (15), both encode functions that can serve to buffer increases in the oxidizing potential inside the cell. Increased OSH1 transcription leads to elevated levels of glutathione while enhanced TRX2 synthesis produces more thioredoxin. Both of these molecules are important intracellular reductants that serve to maintain the normal reducing environment of the cell (35, 36).

Detailed studies in animal cells have provided evidence that NF-κB and AP-1 are likely to serve as a pair of primary and secondary response factors during oxidative stress. NF-κB DNA binding activity is rapidly and strongly induced within 60 min of exposure to H₂O₂ but is repressed by antioxidants (6). Interestingly, AP-1 DNA binding activity is weakly induced by H₂O₂ but is highly responsive to antioxidants such as pyrrolidine dithiocarbamate (6) or thioredoxin (7). The role of yAP-1 in S. cerevisiae appears more similar to that of NF-κB. Oxidants strongly induce yAP-1 transactivation while evidence has been obtained by others that antioxidants suppress yAP-1 transactivation (37). The regulation of yAP-1 by oxidative stress exhibits both positive and negative components. These differing components are defined mutationally by the relative response of a given mutant to either diamide or H₂O₂ exposure. The complex nature of the regulation of yAP-1 in response to oxidative stress is most clearly illustrated by the yAP-1Δ220–335 protein. This mutant protein exhibits greater than wild-type levels of diamide tolerance and ARE-TRP5-lacZ expression. However, the same mutant factor exhibits the opposite behavior in response to H₂O₂. The yAP-1Δ220–335 factor is less effective than wild-type yAP-1 in correcting the H₂O₂ hypersensitivity of a Δyap1 strain and shows a defect in stimulating ARE-TRP5-lacZ expression in response to H₂O₂. These data indicate that this segment of the yAP-1 protein serves as a site for negative control of function during diamide-induced stress but acts as a positive regulatory site upon H₂O₂-induced oxidative challenge.

A second region of yAP-1 also exhibits this differential regulatory functionality. The site 3 CSE mutant (CSE629AAA) is more effective than the wild-type protein in terms of conferring diamide tolerance to the Δyap1 strain and activating diamide-induced ARE-TRP5-lacZ expression. However, this same mutant protein is defective in its ability to correct the H₂O₂ hypersensitivity of a Δyap1 strain although still producing high levels of ARE-TRP5-lacZ-dependent β-galactosidase activity. There are two likely reasons for this discrepancy between H₂O₂ resistance and transactivation mediated by the CSE629AAA mutant protein. Since the H₂O₂-dependent transactivation of the CSE629AAA mutant factor is assessed after 1.5 h of exposure to the oxidant, it is possible that this is insufficient time for the high levels of pre-existing β-galactosidase to turn over and exhibit the expected reduction in activity relative to the normal factor. Owing to the extremely stable nature of E. coli β-galactosidase expressed in yeast (t½ > 20 h (34)), this is a strong possibility. A second possibility is that the CSE629AAA mutant protein is not normally able to activate transcription of

| Site | Site 3 | Site 1,2 | Site 1,3 | Site 2 | Site 2,3 |
|------|--------|----------|----------|--------|----------|
| YPD  |        |          |          |        |          |
| 1.2 μM Diamide | | | | | |
| 1.75 μM H₂O₂ | | | | | |

**FIG. 6. Western of CSE mutant protein levels.** Protein extracts were prepared from cells grown under nonstressed (-) or diamide-treated (+) conditions. 100 μg of protein extract from each condition was electrophoresed on SDS-PAGE, transferred to nitrocellulose, and probed with the anti-yAP-1 antiserum. Molecular mass standards are indicated on the left along with the position of the cross-reacting protein detected by the anti-yAP-1 antiserum (NS). The yAP-1 gene was carried on the low-copy plasmid pRS316. The mutant forms of yAP-1 are indicated by the CSE site that is lost in each variant.

**TABLE III Mutant forms of yAP-1 lacking the normal complement of CSE repeats are defective in oxidative stress regulation**

| YAP1 allele present | ARE-TRP5-lacZ Expression (units/μg) |
|---------------------|-------------------------------------|
| No stress           | Diamide                             |
| Wild-type           |                                    |
| Site 1              | 140 ± 24                            |
| Site 2              | 1325 ± 280                          |
| Site 1,2            | 18 ± 3                              |
| Site 1,3            |                                    |
| Site 1,3            |                                    |
| Site 1,3            |                                    |
| Site 2,3            | 846 ± 236                           |

a SM12 cells were transformed with low-copy plasmids expressing the indicated forms of yAP-1. Appropriate transformants were grown in the absence of stress (no stress) or subjected to diamide-induced (diamide) or H₂O₂-induced (H₂O₂) oxidative stress. ARE-dependent β-galactosidase activities were determined for each transformant.

b Wild-type or mutant versions of the YAP1 gene were carried on the low-copy plasmid pRS316. The mutant forms of YAP1 are indicated by the CSE site that is lost in each variant.

dSM12 cells were transformed with low-copy plasmids expressing the various CSE alanine scanning mutant forms of yAP-1. Selected transformants were grown in minimal medium and processed for spot test analysis as described in the legend to Fig. 3. The CSE mutation present in each form of yAP-1 is listed to the left. Oxidative stress agents were added to YPD medium at the indicated concentrations.
the relevant target gene(s) for $H_2O_2$ resistance although it is capable of activating the ARE-TRP5-lacZ reporter gene. We view this as less likely since all mutant yAP-1 derivatives we have examined exhibit strong correlation between the ability to activate the ARE-TRP5-lacZ reporter gene and the ability to confer resistance to cadmium, cycloheximide, diamide, and $H_2O_2$, with the exception of the CSE629AAA mutant protein and $H_2O_2$ resistance.\(^4\) Discrimination between these and other possible explanations requires further experimentation. An unexpected outcome of these studies is the finding that the same mutant derivative of yAP-1 behaves differently in response to two different oxidants, $H_2O_2$ and diamide. This result suggests that the stresses generated by each type of oxidant are perceived differently by yAP-1. An important issue raised by this observation is the nature of the signal that leads to activation of yAP-1 during oxidative stress. Diamide acts to both deplete glutathione pools and oxidize thiol groups (38) while $H_2O_2$ has several effects including lipid peroxidation, protein oxidation, and DNA damage (39). Although both of these oxidants elicit oxidative stress, their respective intracellular effects are non-identical. Perhaps the transcriptional regulatory properties of yAP-1 vary in response to the type of oxidative stress to which the cell is subjected. Confirmation of this suggestion awaits identification of the relevant downstream target genes of yAP-1.

The possibility that cysteine residues are involved in control of yAP-1 function is interesting in light of studies in E. coli where several different oxidant-sensitive transcription factors use cysteines as part of their redox sensing apparatus. Both SoxR and FNR contain cysteine-bound iron-sulfur clusters that are essential for their function (40–43). OxyR contains six cysteine residues, only one of which is required for regulation of the pAK plasmids and David Katzmann, Sean Coleman, Drs. Jan Fassler, and Wayne Johnson for critical reading of this manuscript.

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\(^4\) S. Steggerda, unpublished observations.
\(^5\) S. Coleman, unpublished data.