The Redox Domain of the Yap1p Transcription Factor Contains Two Disulfide Bonds†

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ABSTRACT: The subcellular localization of the *Saccharomyces cerevisiae* transcription factor Yap1p is regulated by oxidation and reduction. We purified Yap1p from yeast and characterized its properties in vitro. Electrophoretic mobility shift assays showed that the purified protein can specifically bind the *TRX2* target promoter. Yap1p was purified under reducing conditions, but removal of reducing agents resulted in the formation of an oxidized Yap1p species with properties similar to in vivo oxidized Yap1p. MALDI-TOF mass spectrometry analysis revealed that the oxidized form of Yap1p contains two disulfide bonds between C303−C598 and C310−C629. A stable domain of ∼15 kDa was detected upon limited proteolysis of oxidized but not reduced Yap1p. This Yap1p protease resistant domain was purified, and MALDI-TOF mass spectrometry analysis showed that it was comprised of two separate cysteine-containing peptides of Yap1p. These peptides are separated by 250 amino acids and are joined by the C303−C598 and C310−C629 disulfide bonds. Taken together, these data suggest that the domain that controls Yap1p subcellular localization is modular and contains a redox center comprised of four cysteine residues.

All aerobically growing organisms suffer from oxidative stress caused by increased levels of reactive oxygen species such as superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical, and alkyl hydroperoxides (*1*, *2*). To defend against oxidative stress, organisms have evolved a class of redox-sensing proteins whose activities are modulated by reactive oxygen species or by changes in the thiol:disulfide ratio.

Most of the proteins shown to be regulated by oxidation and reduction are involved in transcriptional control such as RsrA, OxyR, CtrJ/PpsR, and OhrR (*3*−*6*), though the activities of Hsp33, a heat shock protein in *Escherichia coli*, and RPTPα, a mammalian receptor protein−tyrosine phosphatase, also have been found to be redox regulated (*7*, *8*). In addition, enzymes that were once thought to only serve as antioxidants, such as Cys-2 Prxs and Gpx3p, now appear to be redox regulated and play a role in H_2O_2 signal transduction (*9*, *10*). The defining features of redox-regulated proteins are conserved cysteine residues that are critical for the activities of these proteins.

The budding yeast *Saccharomyces cerevisiae* has a complex adaptive response to oxidative stress that involves the yeast activator protein 1 (Yap1p) (*1*). Whole genome expression analysis by two-dimensional protein gels and DNA microarrays have shown that Yap1p regulates the expression of as many as 70 genes in response to treatment with H_2O_2 (*11*, *12*). Since Yap1p-DNA binding does not increase in response to oxidative stress, it was suggested that Yap1p activation might be due to posttranslational changes (*13*). Confocal microscopy studies of Yap1p fused to the green fluorescent protein (GFP)[1] revealed that the transcription factor localizes to the nucleus in response to oxidative stress (*13*, *14*). Nuclear export is mediated by Crm1p and is redox dependent, and the proposed nuclear export signal (NES) is located between cysteine residues C598 and C629 (*14*, *15*) (Figure 1). All current models of Yap1p regulation suggest that oxidative stress results in the oxidation of one or more cysteines in Yap1p. These modifications are proposed to effectively shield the NES and disrupt the Yap1p−Crm1p protein−protein interaction. Yap1p accumulates in the nucleus, and gene expression is induced.

Yap1p contains six cysteine residues, five of which are conserved among Yap1p homologues found in other yeast species (Figure 1). There have been several studies of Yap1p cysteine mutants (*13*, *14*, *16*−*18*). The earliest Yap1p mutational studies showed that cysteines C598, C620, and C629 in a domain referred to as the C-terminal cysteine-rich domain (c-CRD) were necessary for Yap1p-dependent gene activation and resistance to H_2O_2 (*16*, *19*). Cysteine C303 in a second domain of Yap1p, referred to as the N-terminal cysteine-rich domain (n-CRD), later also was found to be required for H_2O_2 resistance (*17*). Recent work

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[1] Abbreviations: BME, β-mercaptoethanol; CRD, cysteine-rich domain; c-CRD, C-terminal cysteine-rich domain; EMSA, electrophoretic mobility shift assay; Endo Lys-C, endopeptidase Lys-C; GFP, green fluorescent protein; IAA, iodoacetamide; ICAP, inductively coupled argon plasma; ICP-MS, inductively coupled plasma mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; n-CRD, N-terminal cysteine-rich domain; NES, nuclear export signal; PAR, 4-(2-pyridylazo)-resorcinol; *ptrx2*, *TRX2* promoter; *ptrx2m*, mutant *TRX2* promoter; TFA, trifluoroacetic acid; Yap1p-RD, Yap1p redox domain.

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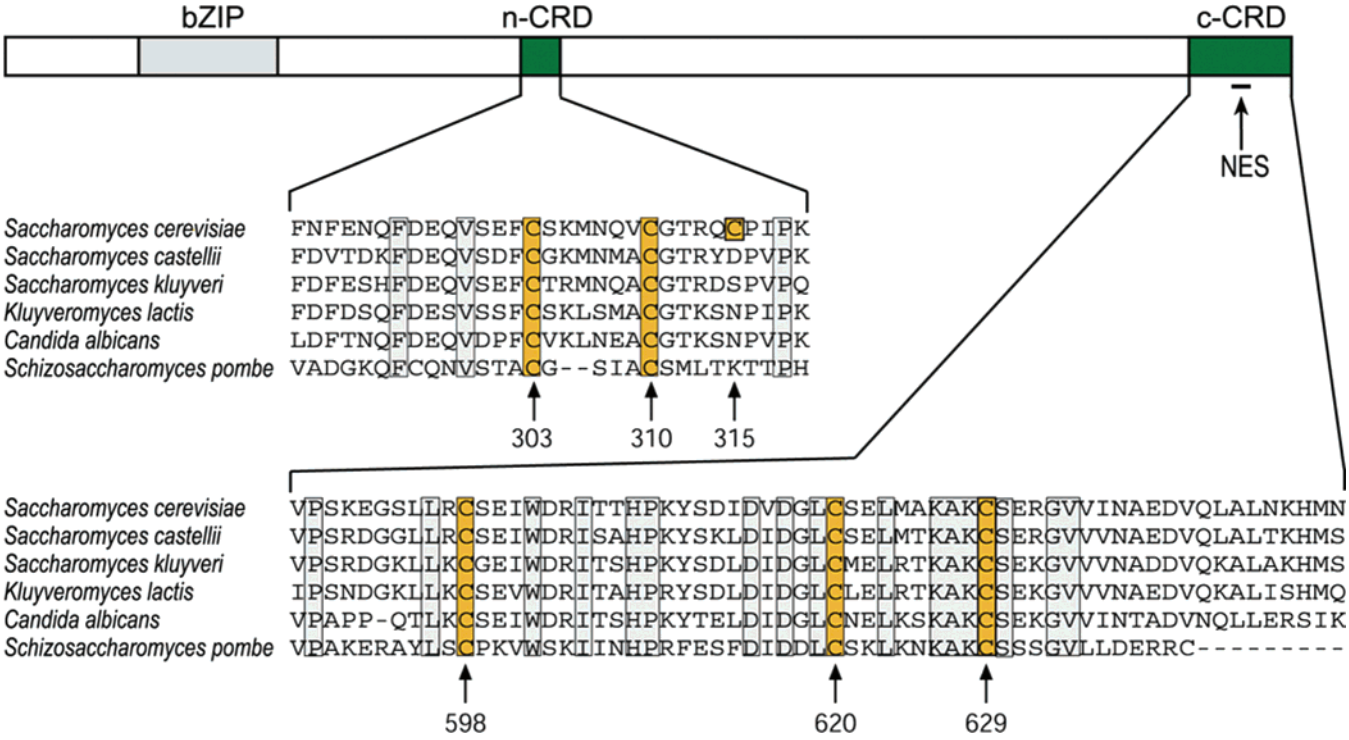

FIGURE 1: Alignment of six yeast Yap1p homologues. Yap1p homologues have three conserved regions: the basic leucine zipper DNA binding domain and the N-terminal and C-terminal cysteine-rich domains (n-CRD and c-CRD). Among the six Yap1p homologues there are five conserved cysteine residues, two in the n-CRD and three in the c-CRD. The NES is embedded within the c-CRD.

has demonstrated that, in cell extracts, oxidized Yap1p migrates faster than the reduced form of the protein on an SDS−PAGE gel. On the basis of this assay, the C303A and C598A mutations abolished Yap1p oxidation, and the C310A and C629A mutations impaired Yap1p oxidation (*18*). Interestingly, however, the expression of the Yap1p target *TRX2* in response to H_2O_2 is attenuated to a similar degree in C303A, C310A, C598A, and C629A mutants. In addition, H_2O_2 tolerance assays showed that C303, C310, C598, and C629 are critical for H_2O_2 resistance, while C315 and C620 are dispensable. Although different studies have implicated different cysteines, all findings are consistent with the idea that the activation of Yap1p occurs through the oxidation of one or more of its conserved cysteine residues.

Despite extensive mutational studies there have been very few biochemical studies of the purified full-length Yap1p. The c-CRD regions of Yap1p and its homologue Pap1p have been examined in vitro, but biochemical studies of the full-length protein have been lacking (*20*, *21*). In this study, we examined the structure and function of full-length Yap1p expressed in and purified from *S. cerevisiae*. We have identified two disulfide bonds that form upon oxidation of Yap1p in vitro. In addition, we have biochemically dissected the oxidized form of the protein and discovered a novel protease-resistant domain in Yap1p that is comprised of the n-CRD and c-CRD regions of the protein joined by the two disulfide bonds.

MATERIALS AND METHODS

Expression of (His)$_6$-Yap1p in Yeast. A recombinant (His)$_6$-Yap1p fusion protein was overexpressed in *S. cerevisiae* using the pYES2/NT-C vector system (Invitrogen). The *YAP1* gene was amplified from the plasmid LDB462 (*14*) and subcloned into the vector pYES2/NT-C using *Eco*RI and *Not*I restriction sites to create the plasmid pYMJW3. A *yap1* deletion strain, YMJW4, was made by replacing the *YAP1* gene with a kanamycin cassette in the protease-deficient *S. cerevisiae* strain c13-ABYS-86 (*22*). YMJW4 then was transformed with the (His)$_6$-Yap1p expression plasmid pYMJW3, and transformants were selected for on minimal media minus uracil (*23*). Baffled shake flasks (4 L) containing 1 L of minimal media (6 L total) were inoculated with 10 mL of saturated culture and were incubated overnight. At an OD_{600} of ∼0.8−1.0, (His)$_6$-Yap1p expression was induced for 4 h with the addition of solid galactose to a final concentration of 2% (w/v). Cell pellets were washed with 600 mL of ice-cold H_2O, pelleted by centrifugation, washed with 300 mL of buffer A, which contained 50 mM Hepes, pH 8.0, 300 mM KCl, 5 mM $MgCl_2$, 10% glycerol, and 10 mM BME, pelleted by centrifugation, and immediately frozen at −80 °C. Approximately 40 g of yeast (wet cell weight) was recovered from 6 L of culture.

Purification of (His)$_6$-Yap1p. All purification steps were performed at 4 °C, and all buffers contained 10 mM BME. Yeast cell pellets were thawed and resuspended in buffer A that contained 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma) and protease inhibitor complete EDTA-free tablets (Roche) in a final volume of 80 mL. To efficiently obtain (His)$_6$-Yap1p extracts, cells were passed through a high-pressure cell disruptor (Constant Systems Ltd.) operating at 35 kpsi. The cell disruptor resulted in cell breakage efficiencies of greater than 90%. Buffer A with 1 mM PMSF and protease tablets was added to bring the volume to ∼200 mL, and the extract was clarified by centrifugation (40000*g*, 1 h). A Ni^{2+} affinity column was prepared with Ni^{2+}-NTA resin (Qiagen) and was equilibrated with 10 column volumes of buffer A plus 20 mM imidazole. The clarified extract was diluted 1:1 with buffer A to ∼400 mL, imidazole was added

to final concentration of 20 mM, and the sample was passed over the Ni^{2+} affinity column at a 0.5 mL/min flow rate. After binding the column was washed with 200 mL of buffer A plus 20 mM imidazole followed by 300 mL of buffer A plus 1 M KCl and 25 mM imidazole. $(His)_6$-Yap1p was eluted from the column in buffer A plus 150 mM imidazole. The Ni^{2+} $(His)_6$-Yap1p fractions were diluted 1:10 with 25 mM Hepes, pH 8.0, and loaded onto a Mono Q column (Amersham Biosciences) equilibrated with 25 mM Hepes, pH 8.0, 50 mM KCl, and 5% glycerol. $(His)_6$-Yap1p was eluted with a linear KCl gradient. Mono Q fractions containing $(His)_6$-Yap1p were pooled (~10 mL), diluted 1:5 with 25 mM Hepes, pH 7.0, loaded onto a Hi-trap heparin column, and eluted with a linear KCl gradient. The fractions containing $(His)_6$-Yap1p were immediately frozen at −80 °C until further use.

All concentrations of $(His)_6$-Yap1p were determined using the Lowry assay. A sample of purified $(His)_6$-Yap1p was dialyzed into H_2O and sent for quantitative amino acid analysis, and the results from this analysis were used to determine the Lowry assay correction factor for $(His)_6$-Yap1p.

Oxidation and Reduction of Yap1p in Vitro and in Vivo. Purified $(His)_6$-Yap1p was oxidized by dialysis into 25 mM Tris, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$. Dialysis included three buffer changes and was carried out at 4 °C over a period of 18 h. Purified oxidized $(His)_6$-Yap1p was reduced using *S. cerevisiae* Trx2p (Calbiochem). Concentration ratios of 1:1 to 1:20 $(His)_6$-Yap1p:Trx2p were prepared and incubated for 20 min at 25 °C. Samples from untreated and H_2O_2-treated cells were obtained using established procedures (*18*). Briefly, a strain, YOR205, that contained three HA-tag fusions to the N-terminus of the *YAP1* gene (O. Carmel-Harel, unpublished data) was grown to an OD_{600} of 0.4 and left untreated or treated with 400 μM H_2O_2 for 10 min. All samples were run on an 8% SDS−PAGE gel under reducing and nonreducing conditions. The gels were Western blotted and probed with anti-6×His (Clonetech) and anti-HA (Roche) monoclonal antibodies according to published procedures.

Yap1p Electrophoretic Mobility Shift Assay (EMSA). Purified $(His)_6$-Yap1p was oxidized by dialysis and frozen at −20 °C until use. The following pair of oligonucleotides corresponding to the Yap1p binding site in the *TRX2* promoter and the mutated form of the promoter were synthesized with a 5′ FAM label: CTCTTTTCTTACTAAGCGCG (*ptrx2*); CTCTTTTCTCTAGAAGCGCG *(ptrx2m*) (Invitrogen) (*24*). The primers were annealed with unlabeled complementary sequences and checked on a native 8% acrylamide gel for proper annealing. Low ionic strength gels for EMSA were prepared and run following standard protocols (*25*). EMSA sample volumes were 15 μL and contained 1 pmol of DNA probe, 20 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC) (Sigma). For EMSA assays using reduced $(His)_6$-Yap1p, 2 mM DTT was included in the binding reactions. Samples were incubated in the dark for 20 min at room temperature, and gels were run at 4 °C for 45 min at 200 V. EMSA gels were visualized with a Typhoon fluorescent scanner (Amersham Biosciences) with excitation at 532 nm. Images were analyzed using ImageQuant software (Amersham Biosciences).

Disulfide Bond Mapping. Oxidized $(His)_6$-Yap1p was digested overnight with endoproteinase Lys-C (Endo Lys-C) (Roche) in the presence of 10 mM iodoacetamide (IAA) at 37 °C. Half of the sample was reduced with 10 mM DTT at 37 °C for 1 h. Samples were acidified with trifluoroacetic acid (TFA) (0.1% final concentration), and Yap1p peptides were exchanged into a 50:50 solution of 0.1% TFA:acetonitrile with a Zip-Tip_{18} (Millipore). Peptide samples were mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid, and 1 μL was spotted on a sample plate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-STR (Perceptive Biosystems). Spectra were collected in the linear mode and calibrated internally using Yap1p peptides. Spectra were analyzed using the program Data Explorer (Perceptive Biosystems).

Limited Proteolysis of (His)$_6$-Yap1p. The redox domain of $(His)_6$-Yap1p (Yap1p-RD) was prepared by digesting oxidized $(His)_6$-Yap1p with limiting amounts of trypsin. Oxidized $(His)_6$-Yap1p was reacted with trypsin (200:1 w/w ratio) at 37 °C for 4 h. The reaction was stopped by the addition of 1 mM PMSF and diluted 1:4 with 25 mM Tris, pH 7.5. The $(His)_6$-Yap1p digestion was loaded onto a Mono Q column equilibrated with 25 mM Tris, pH 7.5. Yap1p-RD was eluted with a linear KCl gradient over 30 min ($[KCl]_f$ = 0.5 M). Mono Q fractions containing Yap1p-RD were pooled, washed three times with 25 mM Tris, pH 7.5, and 100 mM KCl, concentrated in a Centricon YM-3, and stored at −80 °C for further analysis. The identities of the peptides comprising Yap1p-RD were identified with MALDI-TOF MS using sinapinic acid as the matrix. The spectra were calibrated with external standards. Identification of the disulfide-bonding pattern of Yap1p-RD was performed in a manner similar to that of full-length $(His)_6$-Yap1p.

Fluorescence Spectroscopy. Fluorescence measurements were performed on Fluoromax-3 (Johin Yeon Horida). Samples were excited at 280 nm, and the fluorescence emission spectra were collected from 280 to 400 nm. $(His)_6$-Yap1p was at a concentration of 1 μM and in 25 mM Tris, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$. Reduced $(His)_6$-Yap1p contained 5 mM DTT.

(His)$_6$-Yap1p Metal Analysis. Purified $(His)_6$-Yap1p was sent for inductively coupled plasma mass spectrometry (ICP-MS) metal analysis at the University of Georgia. Samples of reduced and oxidized $(His)_6$-Yap1p were dialyzed extensively against metal-free buffer and frozen at −70 °C. Metal-free buffer samples were sent for ICP-MS as controls. To test specifically for the presence of zinc in purified $(His)_6$-Yap1p, Yap1p samples were treated with the zinc-specific dye 4-(2-pyridylazo)resorcinol (PAR) (Sigma) (*26*, *27*). $(His)_6$-Yap1p treated with 100 μM PAR was analyzed with UV−visible spectroscopy. A Zn-PAR standard curve was used for calibration.

RESULTS

Purification of (His)$_6$-Yap1p. To allow biochemical characterization of the Yap1p transcription factor, the full-length protein was purified. Since refolding large non-native proteins expressed in *E. coli* into the correct active form can be difficult, we chose to isolate Yap1p from *S. cerevisiae*, its native organism. Quantities sufficient for biochemical

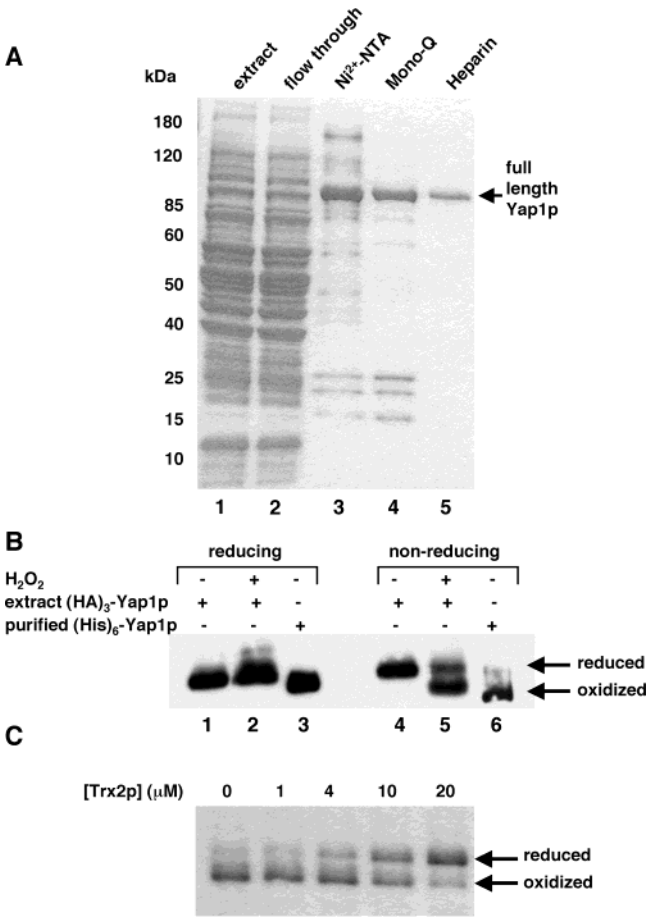

FIGURE 2: Oxidation and reduction of in vitro purified $(His)_6$-Yap1p. (A) Coomassie blue stained SDS−PAGE gel of the purification of $(His)_6$-Yap1p from *S. cerevisiae*. Lanes: 1, crude yeast extract; 2, flow through from the Ni^{2+}-NTA column; 3, 150 mM imidazole fraction from the Ni^{2+}-NTA column; 4, Mono Q $(His)_6$-Yap1p fraction; 5, Hi-trap Heparin $(His)_6$-Yap1p fraction. (B) Western blot of in vivo $(HA)_3$-Yap1p isolated from untreated and H_2O_2-treated cells and in vitro purified $(His)_6$-Yap1p separated on reducing (lanes 1−3) and nonreducing (lanes 4−6) SDS−PAGE gels. Lanes: 1, $(HA)_3$-Yap1p untreated; 2, $(HA)_3$-Yap1p + H_2O_2; 3, $(His)_6$-Yap1p; 4, $(HA)_3$-Yap1p untreated; 5, $(HA)_3$-Yap1p + H_2O_2; 6, $(His)_6$-Yap1p. (C) Incubation of $(His)_6$-Yap1p with increasing concentrations of Trx2p.

experiments were obtained by placing a $(His)_6$ tag on the N-terminus of Yap1p and expressing the protein in a protease-deficient *yap1* deletion strain. Compared to the parent strain, the overexpression strain was more resistant to H_2O_2 and showed higher expression of a Yap1 target gene fusion, indicating that overexpressed $(His)_6$-Yap1p is functional (data not shown). An SDS−PAGE gel showing the progress of $(His)_6$-Yap1p purification (Figure 2A) demonstrated that the protein is ∼85% pure after elution from the Ni^{2+}-NTA column. To remove copurifying proteins, $(His)_6$-Yap1p was further purified on a Mono Q column and then a Hi-trap heparin column. $(His)_6$-Yap1p in the pooled fractions from the heparin column was >95% pure as judged by a Coomassie blue stained SDS−PAGE gel (Figure 2A). The yield of purified $(His)_6$-Yap1p was 0.15 mg/L of yeast culture.

Redox Activity of $(His)_6$-Yap1p. To preserve Yap1p in its reduced form, every step of the purification of $(His)_6$-Yap1p included BME. Previous work showed that in vivo oxidation of Yap1p with H_2O_2 results in a faster migrating band on an

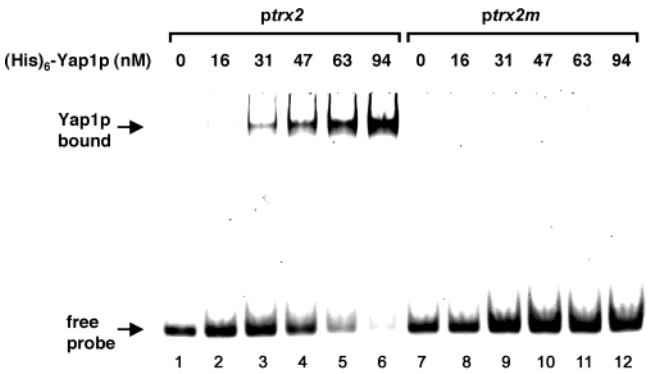

FIGURE 3: Characterization of in vitro purified $(His)_6$-Yap1p. Binding of $(His)_6$-Yap1p to the *TRX2* promoter was assayed by incubating increasing concentrations of purified oxidized Yap1p with 5′-FAM labeled p*trx2* (lanes 1−6) or p*trx2m* (lanes 7−12).

SDS−PAGE gel run under nonreducing conditions, and this band reverts back to a slower migrating one when reducing agent is present (*18*). Purified $(His)_6$-Yap1p was exchanged into buffer that did not contain reducing agent and analyzed on SDS−PAGE gels together with cell extracts from a strain expressing $(HA)_3$-Yap1p which was left untreated or treated with H_2O_2 (Figure 2B). Under reducing conditions (Figure 2B, lanes 1 and 2), the $(HA)_3$-Yap1p from both the H_2O_2-treated and untreated cells migrated identically. The $(His)_6$-Yap1p protein appeared to migrate slightly faster than the $(HA)_3$-Yap1p protein under these conditions (Figure 2A, lane 3). This was not surprising given that the two proteins contain different N-terminal tags. Under nonreducing conditions, the $(HA)_3$-Yap1p from cells treated with H_2O_2 migrates faster than the untreated one (Figure 2A, lanes 4 and 5), again similar to previous data. Under nonreducing conditions the $(His)_6$-Yap1p protein also migrates faster when compared to reduced $(His)_6$-Yap1p (Figure 2A, lane 6). These results suggest that $(His)_6$-Yap1p is oxidized similarly to $(HA)_3$-Yap1p isolated from cells treated with H_2O_2. Therefore, we will refer to and consider this form of $(His)_6$-Yap1p as oxidized.

Genetic and biochemical data have suggested that Yap1p is reduced by Trx2p in vivo (*18*, *28*, *29*). To test whether Trx2p could reduce $(His)_6$-Yap1p in vitro, purified *S. cerevisiae* Trx2p was added to oxidized $(His)_6$-Yap1p in stoichiometric amounts. The addition of Trx2p led to almost complete reduction of oxidized $(His)_6$-Yap1p (Figure 2C), providing additional evidence that the purified oxidized $(His)_6$-Yap1p behaves like the in vivo oxidized form of Yap1p.

DNA Binding of $(His)_6$-Yap1p. Electrophoretic mobility shift assays were performed with purified $(His)_6$-Yap1p to evaluate its DNA binding activity. Two fluorescently labeled oligonucleotide probes were synthesized for these experiments. One consisted of the first Yap1p binding site of the *TRX2* promoter (p*trx2*). A second probe containing point mutations in the Yap1p binding site was used as a control (p*trx2m*) (*24*, *30*). Varying concentrations of oxidized $(His)_6$-Yap1p were incubated with p*trx2* and p*trx2m.* As expected, $(His)_6$-Yap1p tightly bound p*trx2* with a K_D of 45 nM and did not bind p*trx2m* (Figure 3). This dissociation constant is similar to the 35 nM value measured for Gcn4p, another yeast transcription factor containing a bZIP DNA binding domain (*31*, *32*). EMSA experiments comparing oxidized and

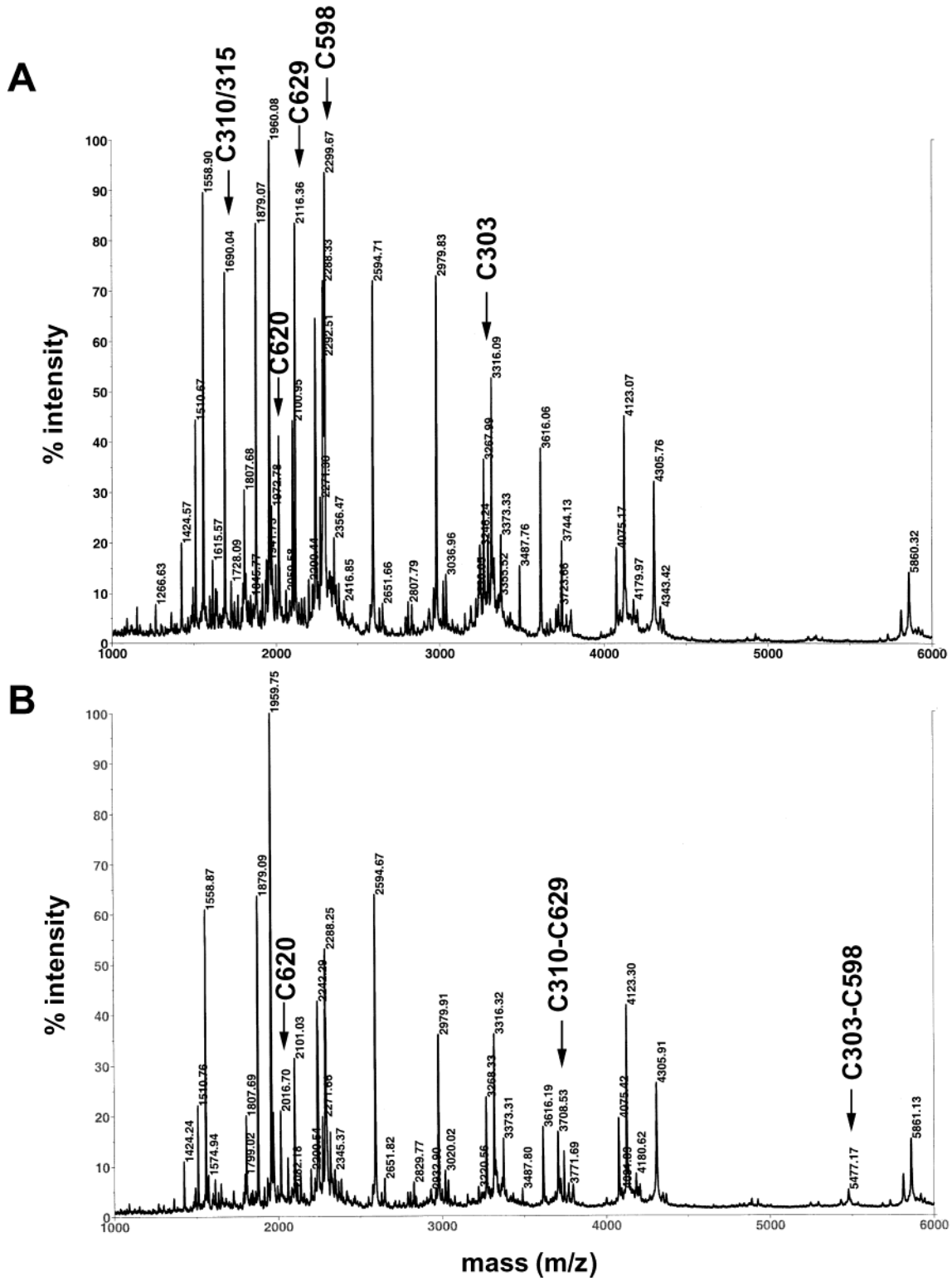

FIGURE 4: MALDI-TOF MS of reduced (A) and oxidized (B) $(His)_6$-Yap1p. Oxidized $(His)_6$-Yap1p was digested overnight with endoproteinase Lys-C (Roche) in the presence of 10 mM IAA at 37 °C. Half of the sample was reduced with 10 mM DTT at 37 °C for 1 h. Cysteine-containing peptides are indicated with arrows.

reduced $(His)_6$-Yap1p showed that the two forms of the purified protein have the same dissociation constants. This result agreed with previous experiments showing that Yap1p DNA binding affinities are not different in extracts prepared from untreated and H_2O_2-treated cells (*13*).

Status of Cysteines in Oxidized and Reduced $(His)_6$-Yap1p. We wanted to investigate the differences in the cysteine oxidation states of oxidized and reduced forms of $(His)_6$-Yap1p. Thus $(His)_6$-Yap1p was completely digested with Endo Lys-C, and the digest was analyzed with MALDI-TOF mass spectrometry. Endo Lys-C only cleaves on the C-terminal side of lysine residues and yielded simpler mass spectra than other proteases such as trypsin and chymotrypsin. Endo Lys-C also had the advantage that it cleaved between C303, C310, C598, C620, and C629. To obtain consistent digestion patterns for both forms of the protein,

Table 1: MALDI-TOF MS of Yap1p Cysteine-Containing Peptides

Yap1p residues	cysteine(s)	expected mass (Da)[a]	observed mass (Da)	
			reduced[a]	oxidized[a]
279−305	303	3294.48	3294.87	
306−319	310, 315	1690.02	1690.03	
592−610	598	2299.62	2299.71	
611−628	620	2016.30	2016.68	2016.76
629−647	629	5516.41	2116.39	
306−319, 629−647	310, 315, 629	3690.30[b]		3708.71[b]
279−305, 592−610	303, 598	54477.99		5477.50

[a] Expected and observed peptide masses are based on alkylation of all cysteine residues resulting in an increase in 57 Da per cysteine. [b] Since there are three cysteines in these peptides, the expected mass was calculated to include one disulfide bond and one alkylation. The observed mass was +18.4 Da higher, corresponding to one BME modification and not alkylation.

oxidized $(His)_6$-Yap1p was digested in the presence of excess IAA so that all free thiols would be trapped in the alkylated form. Half of the reaction then was treated with DTT. The peptides present in both samples were analyzed with MALDI-TOF MS. The resulting spectra were identical except for the differences in the oxidation states of the cysteine-containing peptides (Figure 4). For both reduced and oxidized $(His)_6$-Yap1p, greater than 90% of the expected peptides of the 689 amino acid protein were observed. All of the cysteine-containing peptides were unambiguously identified in both spectra. A majority of the peaks in the oxidized spectra were unchanged by addition of DTT, including the peptide containing C620. The C620-containing peptide was observed in both reduced and oxidized spectra at a masses of 2016.68 and 2016.76 Da, respectively (Table 1). The expected mass of this peptide modified with IAA is 2016.30 Da. Therefore, C620 is a thiol in the oxidized from of $(His)_6$-Yap1p, which correlates with the observations that in vivo C620 is not critical for the Yap1p response to H_2O_2.

In the reduced $(His)_6$-Yap1p spectrum, all of the expected cysteine-containing peptides were observed in their alkylated forms (Figure 4A and Table 1). In the oxidized spectrum the peptides corresponding to alkylated C303, C310/315, C598, and C629 were all not observed (Figure 4B). Instead of the individual cysteine peptides, the oxidized spectrum contained two new peaks at 3708.71 and 5477.50 Da. The absence of four cysteine peptides in the oxidized spectrum and observation of two new peaks suggested that oxidized $(His)_6$-Yap1p contains two specific disulfide bonds. The size of a 5477.50 Da peak matched the expected molecular mass of a 5477.99 Da fragment which corresponds to Yap1p amino acids 279−305 (C303) and 592−610 (C598) joined by a disulfide bond. Reduction of $(His)_6$-Yap1p resulted in the observation of the C303 and C598 peptides, indicating that these cysteines are only available to react with IAA upon reduction with DTT. It also has been shown that C303 and C598 are essential for oxidation of Yap1p in vivo, and this disulfide bond was predicted previously.

The DTT-sensitive peak at 3708.71 Da was 18.4 Da greater than the expected molecular mass of peptides 306−319 (C310/315) and 629−647 (C629) joined by a disulfide bond (3690.30 Da). Because of the lack of a lysine residue in the amino acid sequence between C310 and C315, these two cysteines are not separated by Endo Lys-C. If this peptide

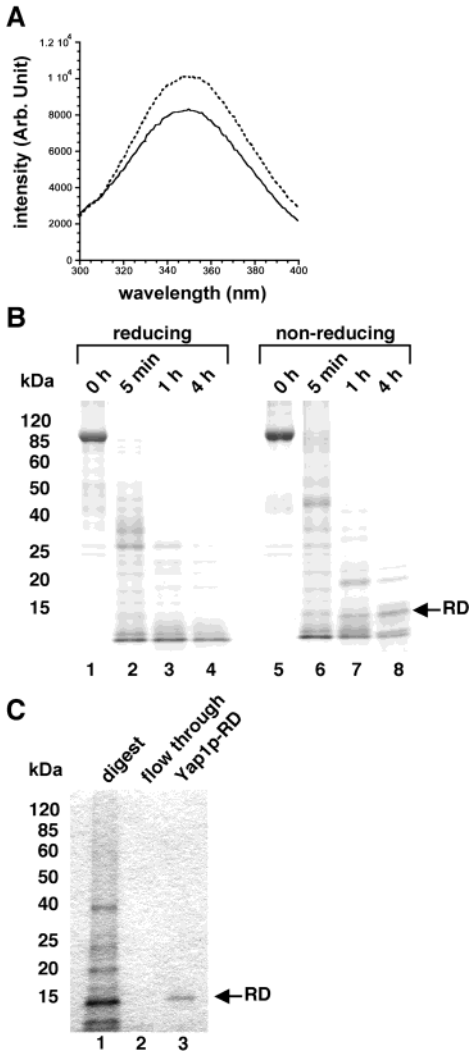

FIGURE 5: Conformational changes between oxidized and reduced $(His)_6$-Yap1p. (A) Fluorescence emission spectra of oxidized (solid line) and reduced (dashed line) $(His)_6$-Yap1p. (B) $(His)_6$-Yap1p digested under reducing (lanes 1−4) or nonreducing (lanes 5−8) conditions with limiting amounts of trypsin (200:1 w/w) over 4 h. Lanes: 1 and 5, predigest; 2 and 6, 15 min; 3 and 7, 1 h; 4 and 8, 4 h. The Yap1p-RD (indicated with an RD) band only appears when Yap1p is digested under nonreducing conditions. (C) Purification of Yap1p-RD on a Mono Q column. Lanes: 1, $(His)_6$-Yap1p digest with trypsin for 4 h; 2, flow through of the digest from the Mono Q column; 3, Yap1p-RD fraction from the Mono Q column.

were involved in a disulfide bond, the remaining cysteine would be expected to be available for alkylation by IAA contributing 57 Da. Instead, there was a peak 18.4 Da higher that expected, possibly due to the presence of a 75.4 Da adduct. A search of the Association of Biomolecular Resource Facilities posttranslational modifications database (http://www.abrf.org/index.cfm/dm.home) revealed that a +76 Da increase could correspond to a BME modification. Therefore, we suggest that the peak at 3708.71 Da is a result of a disulfide bond between C310−C629 with the remaining cysteine C315 modified by BME and not IAA. This is not surprising since $(His)_6$-Yap1p is purified in the presence BME, which has been observed to modify cysteines in other redox-active proteins (*33*). The finding that, upon reduction, the C310−C629 and C315−BME disulfides are reduced and the C310/315 peptide is observed to be alkylated on both cysteines is consistent with the conclusion that C315 contains

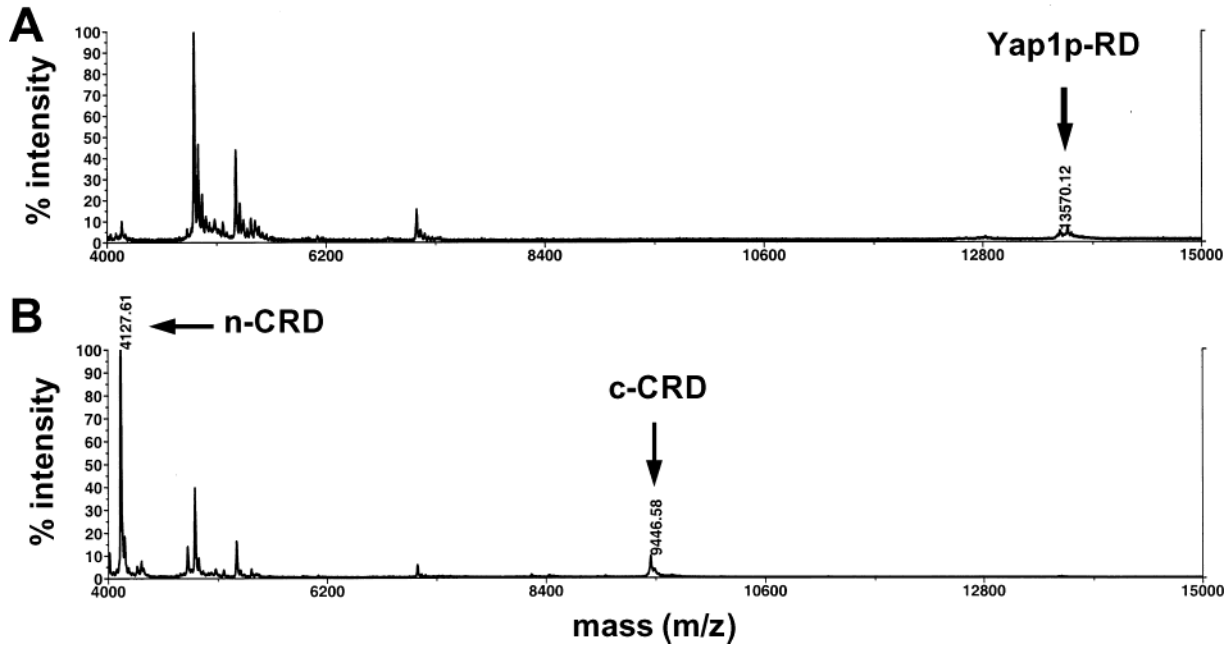

FIGURE 6: MALDI-TOF mass spectra of reduced (A) and oxidized (B) purified Yap1p-RD. Oxidized Yap1p-RD was reduced with 5 mM DTT. The observed masses of the two new peaks in the reduced spectra match the theoretical masses of peptides comprising the n-CRD (4127.48 Da) and c-CRD (9446.46 Da) of Yap1p.

a BME adduct. It is likely that C310 and not C315 is disulfide bonded with C629. C310 is conserved in Yap1p homologues while C315 is not conserved. Furthermore, mutation of C315 does not affect Yap1p's ability to respond to oxidative stress in vivo, while C310 has been demonstrated to be critical for in vivo activity.

Conformational Differences between Oxidized and Reduced Yap1p. The current model for Yap1p regulation suggests that the NES is accessible in the reduced form of the protein and that upon oxidation Yap1p undergoes a conformational change and the NES is no longer accessible. Using the purified forms of oxidized and reduced $(His)_6$-Yap1p, the structural changes in Yap1p in response to oxidation and reduction were investigated. To measure redox-dependent conformational changes in the tertiary structure of $(His)_6$-Yap1p, the fluorescence emission spectra were monitored after excitation at 280 nm. There are three tryptophan residues in Yap1p. Of these, W602 is conserved in Yap1p homologues and is located three residues from C598. There is an ~20% increase in the fluorescence emission intensity between oxidized and reduced $(His)_6$-Yap1p (Figure 5A). A large change in the fluorescence emission is characteristic of a change in the molecular environment of a tryptophan side chain. These results suggest that Yap1p can accommodate multiple conformations that are linked to its redox state.

The proteolytic sensitivity of reduced and oxidized $(His)_6$-Yap1p was monitored using limiting amounts of trypsin and chymotrypsin. Preparations of oxidized and reduced $(His)_6$-Yap1p were digested, and aliquots were removed at various time points and run on nonreducing SDS−PAGE gels (Figure 5B). After 4 h (Figure 5B, lane 8), a band of ~15 kDa was detected in the oxidized but not the reduced samples (Figure 5B, lane 4). This 15 kDa band was observed for up to 24 h. In digests where chymotrypsin was used, a similar redox-sensitive band that migrated at 19 kDa was observed (data not shown). Both the 15 and the 19 kDa band disappeared when the digest from the oxidized sample was run on a reducing gel (data not shown). This observation suggests that the fragment contains at least one disulfide bond. We have termed this fragment the Yap1p redox domain or Yap1p-RD.

Characterization of the Yap1p Redox Domain (Yap1p-RD). To allow for MALDI-TOF MS analysis of the Yap1p-RD, oxidized, full-length $(His)_6$-Yap1p was digested with limiting amounts of trypsin. The entire digestion then was loaded on a Mono Q column, and Yap1p-RD-containing fractions were pooled and concentrated (Figure 5C). The mass spectrum of Yap1p-RD shows a peak at 13570.12 Da, which matches the ~15 kDa band observed on an SDS−PAGE gel (Figure 6A). When this sample is treated with DTT, the peak at 13570.12 disappears, and two new peaks appear at 4127.61 and 9446.58 Da (Figure 6B). The Yap1p sequence was searched for tryptic peptides that matched the 4127.61 and 9446.58 Da peaks. A sequence comprising residues 279−313 was a match to the 4127.61 Da peak. This sequence is known as the n-CRD and includes C303 and C310 but not C315. A sequence comprising residues 565−650 was a match to the 9446.58 Da peak. This sequence is known as the c-CRD and includes C598, C620, and C629. Addition of the n-CRD and c-CRD peptide masses gives a total mass of 13574.19 Da. The difference between this value and the observed mass of 13570.12 is 4.07 Da, which is consistent with the presence of two disulfide bonds in oxidized Yap1p.

To further characterize the Yap1p-RD, the domain was digested to completion with Endo Lys-C and analyzed with and without reduction by MALDI-TOF MS. The individual cysteine residues only were observed in the reduced spectra (Figure 7A). The masses of the cysteine-containing peptides were similar to those in $(His)_6$-Yap1p expect for C310. In Yap1p-RD, C310 has been separated from C315, and the peptide now has a molecular mass of 966.89 Da. In the

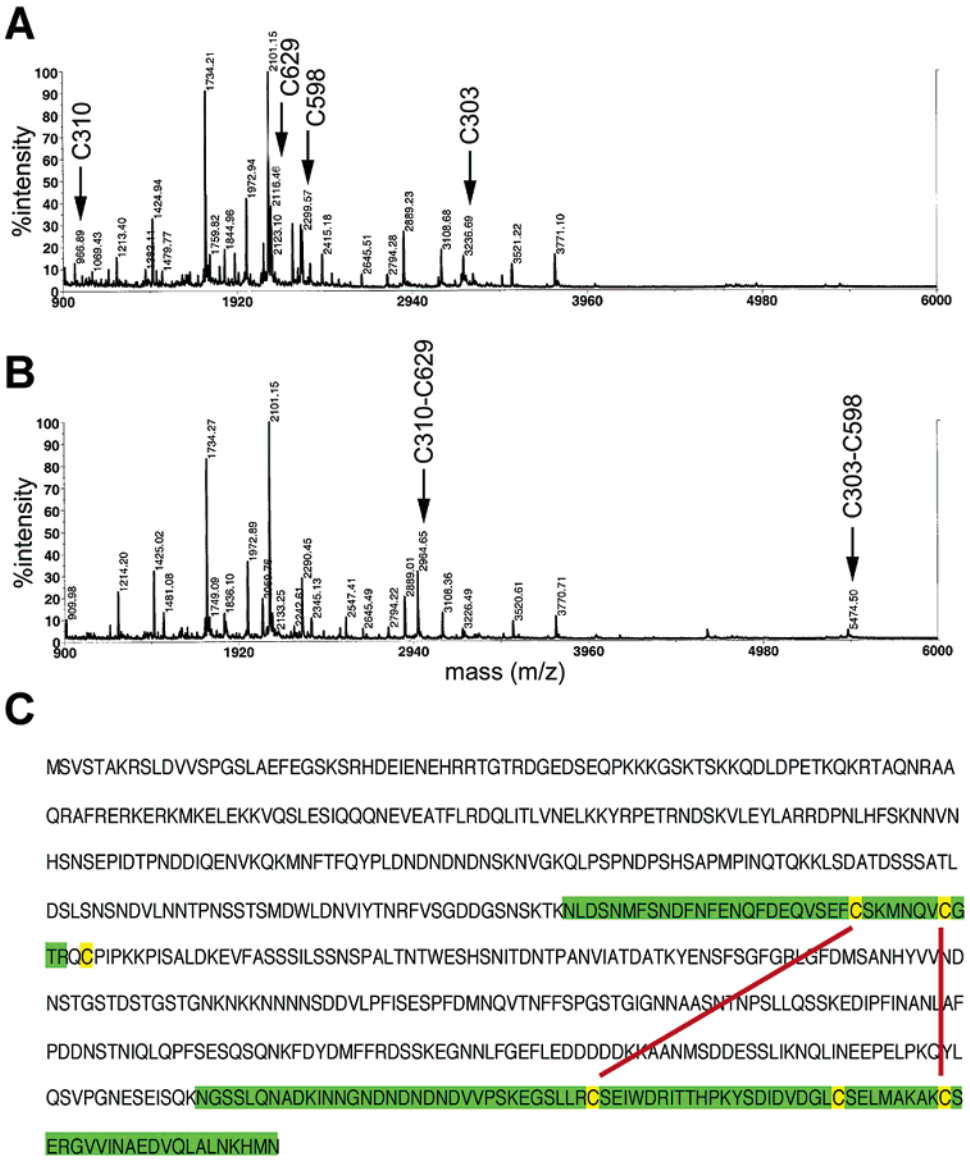

FIGURE 7: Characterization of Yap1p-RD by MALDI-TOF MS. Complete digestion of the purified Yap1p-RD with endoproteinase Lys-C in the presence of 10 mM IAA at 37 °C. Mass spectra of Yap1p-RD treated with 10 mM DTT (A) and oxidized Yap1p-RD (B). The Yap1p-RD spectra show that there are two peaks in the oxidized form of Yap1-RD that disappear upon treatment with DTT. The four individual cysteine peaks are only observed in the reduced spectra. (C) Yap1p sequence showing the mapped Yap1p-RD peptides in green, the cysteine residues in yellow, and the observed disulfide bonds in red.

oxidized Yap1p-RD spectrum, the individual cysteine-containing peptides were not observed, and two new peptides were present at 2964.65 and 5474.50 Da (Figure 7B). The peak at 5474.50 Da matches the molecular mass of peptides corresponding to Yap1p amino acids 279−305 (C303) and 592−610 (C598) joined by a disulfide bond. The peak at 2964.65 Da matches the molecular mass of peptides 306−313 (C310) and 627−647 (C629) joined by one disulfide bond. Taken together, these data are consistent with the conclusion that Yap1p-RD is composed of two distinct peptides from regions of Yap1p separated by 250 amino acids and that the two peptides are joined by disulfide bonds between C303−C598 and C310−C629 (Figure 7C).

(His)$_6$-Yap1p Metal Analysis. Many of the proteins whose activities are regulated by oxidation and reduction of disulfide bonds contain Zn or other metals as part of their redox centers (*4*, *34*). Thus we wanted to determine whether (His)$_6$-Yap1p contained zinc or any other type of transition metal. (His)$_6$-Yap1p samples analyzed with ICP-MS showed the presence of stoichiometric amounts of both zinc and copper. The ICP-MS analysis also was carried out for other transition metals such as Fe, Cr, Co, Ni, and Mn, but these metals were not found to be present. Both oxidized and reduced (His)$_6$-Yap1p contained the same amount of metal. To further examine the zinc content of (His)$_6$-Yap1p, samples of oxidized and reduced (His)$_6$-Yap1p were treated with the zinc-specific dye PAR. Both samples turned orange upon the addition of PAR and showed absorbance peaks at 498 nm, indicating the presence of Zn in the protein samples. On the basis of a Zn standard curve, the zinc to (His)$_6$-Yap1p ratio was 4:1 in both samples. Since Yap1p contained a His$_6$ tag, it is possible that the metal is nonspecifically bound by the tag and not by Yap1p itself. However, inductively coupled argon plasma (ICAP) metal analysis also was carried out on a (His)$_6$-tagged Yap1p-RD derivative isolated from *E. coli* (n-CRD and c-CRD peptides joined by a linker; unpublished data), and no metal was found to be associated with the Yap1p-RD. We believe that, taken together, these data suggest that

although purified $(His)_6$-Yap1p has metals loosely associated with it, the metals are not involved in the redox mechanism.

DISCUSSION

The goal of this study was to investigate the changes that occur in Yap1p upon oxidation. Full-length Yap1p was purified from its native organism and shown to specifically interact with the *TRX2* promoter. We were able to generate oxidized and reduced forms of Yap1p with properties similar to those of Yap1p in cell extracts. MALDI-TOF MS of the oxidized form of Yap1p revealed that it contains two intramolecular disulfide bonds between conserved cysteine residues. The detection of the C303 and C598 disulfide bond confirms and supports extensive mutational data indicating that these residues are an essential part of the Yap1p redox center (*10*, *17*, *18*). The detection of a second disulfide between C310 and C629 correlates with the functional importance and conservation of these residues among Yap1p homologues.

The C303, C310, C598, and C629 residues all are conserved in other Yap1p homologues, suggesting a functional role for each of these cysteines (Figure 1). Other groups performed extensive mutagenesis studies on Yap1p and proposed that Yap1p is regulated by the oxidation of one or more of its cysteine residues (*13*, *16*−*18*). Several investigations indicated that C303, C310, C598, and C629 are all required for the Yap1p response to H_2O_2 (*17*, *18*). Strains expressing C303A, C310A, C598A, and C629A Yap1p mutants have similar phenotypes. All of these mutant strains are unable to grow in the presence of H_2O_2. Furthermore, these strains fail to transcribe *TRX2* on a functionally relevant time scale after H_2O_2 treatment. The Yap1p C303A, C310A, C598A, and C629A mutant proteins also do not become phosphorylated in response to H_2O_2, indicating impaired nuclear accumulation. However, the Yap1p C303A, C310A, C598A, and C629A mutants do exhibit differences in their ability to be oxidized in response to H_2O_2. When Yap1p C303A and C598A mutant strains are treated with H_2O_2 and extracts from these strains are run on nonreducing SDS−PAGE gels, the oxidized form of Yap1p is not observed. When Yap1p C310A and C629A mutant strains are treated with H_2O_2 and extracts from these strains are run on nonreducing SDS−PAGE gels, a mix of oxidized and reduced Yap1p is observed. We suggest that the C310−C629 disulfide bond is unable to form in the C303A and C598A mutants while the C303−C598 disulfide bond is less stable in the C310A and C629A mutants and thus more readily reduced by Trx2p.

Many of the proteins regulated by oxidation and reduction that contain multiple cysteine residues, such as Hsp33 and RsrA, have been shown to contain zinc in their redox-active centers (*4*, *7*). It is proposed that the cysteines in the redox-active center coordinate zinc and are therefore in their thiolate form. Because these cysteines are already deprotonated, they are poised to be oxidized when challenged by reactive oxygen species. The metal analysis of $(His)_6$-Yap1p showed that both copper and zinc were present. However, the $(His)_6$-Yap1p:metal stoichiometry did not change between the oxidized and reduced forms of the protein. Our interpretation of these results is that while metal is present in our preparations of full-length $(His)_6$-Yap1p, metal is not part of the Yap1p redox center. Zinc analysis with PAR showed that samples of purified Yap1p contained Zn that was loosely associated. In addition, ICAP analysis of the Yap1p-RD revealed that it contained no metals. Therefore, we conclude that metals are associated with another region of $(His)_6$-Yap1p and that metal is not part of the redox center of Yap1p.

Recent studies on H_2O_2-mediated signal transduction in *S. cerevisiae* indicate that the proteins Gpx3p and Ybp1p are involved in the oxidation of Yap1p (*10*, *35*). Gpx3p is responsible for formation of the Yap1p C303−C598 disulfide bond and thus regulates Yap1p subcellular localization (*10*). The specific role of Ybp1p in Yap1p H_2O_2-mediated oxidation is unclear, but Ybp1p has been shown to interact with Yap1p, and it has been suggested to act in the same pathway as Gpx3p (*35*−*37*). The contributions of Gpx3p and Ybp1p to C310 and C629 oxidation are not clear. While Yap1p C310A and C629A mutants can react with Gpx3 and form a C303−C598 disulfide bond, available data suggest that the disulfide bond in these mutants is less stable than in the wild-type protein and that the derivatives cannot sufficiently accumulate in the nucleus to elicit a transcriptional response (*18*). Yap1p with two disulfide bonds should be more resistant to reduction in vivo and therefore able to remain oxidized long enough to stay in the nucleus and transcribe its target genes. A potential working model is that, after the formation of the C303−C598 intermolecular disulfide through the Gpx3p/Ybp1p pathway, C310 and C629 form a second disulfide bond under oxidizing conditions. It is also possible that Ybp1p is responsible for the formation of the C310−C629 disulfide, thereby providing alternate pathways for the formation of each disulfide. An oxidized Yap1p containing two disulfides could better shield the NES and confer a "locked" Yap1p structure that is more resistant to reduction. This form of oxidized Yap1p would be able to efficiently accumulate in the nucleus and initiate transcription of its target genes.

Yap1p also has been shown to accumulate in the nucleus in response to other oxidizing agents such as diamide (*13*, *14*). The Yap1p response to diamide is different than its response to H_2O_2 (*16*, *17*). While C303, C310, C598, and C629 are essential for the Yap1p-mediated response to H_2O_2 (*18*), only C620 is required for the response elicited by diamide (*13*). We suggest that Yap1p containing the C303−C598 and C310−C629 disulfide bonds is the active form in cells treated with H_2O_2. However, different combinations of oxidized cysteines and disulfide bonds could lead to Yap1p activation in cells treated with other oxidants.

The model that Yap1p undergoes a conformational change in response to oxidation was supported by two of our observations. First, the intrinsic fluorescence of $(His)_6$-Yap1p changes upon the addition of the reducing agent DTT. Second, the proteolytic sensitivities of reduced and oxidized Yap1p are different. Our results indicate that the formation of disulfide bonds significantly alters the Yap1p structure, making the redox domain less susceptible to protease digestion. We purified and identified the Yap1p protease resistant domain, termed Yap1p-RD, and found it is redox sensitive and composed of the n-CRD and c-CRD joined by the C303−C598 and C310−C629 disulfide bonds. The presence of a distinct domain comprised of the n-CRD and c-CRD, despite a separation of 250 amino acids in the linear

sequence, is supported by the finding that a Yap1p 322−469 deletion mutant strain shows wild-type resistance to oxidants (*16*). We believe that Yap1p-RD comprises the redox center of Yap1p and that the redox-sensing machinery is present in this fragment of Yap1p.

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