The EMBO Journal Vol.16 No.7 pp.1710–1720, 1997

Regulation of yAP-1 nuclear localization in response to oxidative stress

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The *YAP1* gene of *Saccharomyces cerevisiae* encodes a bZIP-containing transcription factor that is essential for the normal response of cells to oxidative stress. Under stress conditions, the activity of yAP-1 is increased, leading to the induced expression of a number of target genes encoding protective enzymes or molecules. We have examined the mechanism of this activation. Upon imposition of oxidative stress, a small increase in the DNA-binding capacity of yAP-1 occurs. However, the major change is at the level of nuclear localization; upon induction the yAP-1 protein relocalizes from the cytoplasm to the nucleus. This regulated localization is mediated by a cysteine-rich domain (CRD) at the C-terminus, its removal resulting in constitutive nuclear localization and high level activity. Furthermore, the CRD of yAP-1 is sufficient to impose regulated nuclear localization of the GAL4 DNA-binding domain. Amino acid substitutions indicated that three conserved cysteine residues in the CRD are essential for the regulation. We suggest therefore, that these cysteine residues are important in sensing the redox state of the cell and hence regulating yAP-1 activity.

Keywords: cysteine/nuclear localization/oxidative stress/transcriptional regulation/*YAP1*

Introduction

All organisms have evolved cellular responses to combat adverse and abrupt environmental changes, which, in most cases, involve the increased synthesis and activation of molecules or enzymes that serve a protective function (Welch, 1993; Mager and Kruijff, 1995; Ruis and Shüller, 1995). Such responses are important for cell survival and as such there has been considerable interest in the nature of the signals and factors that mediate the changes in the expression pattern of the cell. One such response mechanism is triggered by oxidative stress which, through the generation of reactive oxygen species (ROS), can induce considerable macromolecular damage (Halliwell and Gutteridge, 1984). As a result, all aerobic organisms have defence mechanisms to protect against such oxidants. The mechanisms that underlie the oxidative stress response have been well characterized in the prokaryotes *Escherichia coli* and *Salmonella typhimurium* where the transcription factors OxyR and SoxR/SoxS serve to activate the expression of multiple genes that encode proteins or enzymes which can scavenge ROS as well as repair damaged cellular proteins (Farr and Kogoma, 1991). In eukaryotes, however, the mechanisms are far less clear; most progress has been made in budding yeast where the availability of genetic analysis has allowed the identification of some of the cellular products involved (Moradas-Ferreira *et al.*, 1996).

One transcription factor that is important for oxidative stress response in *Saccharomyces cerevisiae* is yAP-1 (Schnell *et al.*, 1992; Hirata *et al.*, 1994; Kuge and Jones, 1994). This factor was initially identified and cloned by virtue of its ability to bind specifically to AP-1 sites. It is a bZIP-containing factor that shows homology within its DNA-binding domain to members of the mammalian Jun family of proteins (Moye-Rowley *et al.*, 1989). The importance of yAP-1 to stress response has been highlighted by at least three different lines of evidence. First, the *YAP1* gene was isolated as a gene which, in high copy number, could confer pleiotropic drug resistance as well as cadmium resistance (Hertle *et al.*, 1991; Hussain and Lenard, 1991; Schnell and Entian, 1991; Wu *et al.*, 1993; Kuge and Jones, 1994). Second, disruption of the *YAP1* gene caused significant increased sensitivity to oxidative stress generated by agents such as H_2O_2 and diamide and to cadmium toxicity (Schnell and Entian, 1991; Kuge and Jones, 1994). Third, the activity of yAP-1 can be induced by oxidative stress generated by H_2O_2, diamide and diethyl maleate (Hirata *et al.*, 1994; Kuge and Jones, 1994). These observations suggested that in response to different stress conditions, induction of yAP-1 resulted in increased expression of crucial target genes important for the cell response. A number of such targets have now been identified, including the *TRX2* gene which encodes thioredoxin that confers increased resistance to oxidative stress (Kuge and Jones, 1994), *YCF1* which encodes an ATP-binding cassette transporter gene essential for cadmium tolerance (Wemmie *et al.*, 1994a), *GSH1* which encodes γ-glutamylcysteine synthetase involved in glutathione biosynthesis (Wu and Moye-Rowley, 1994), *GLR1* which encodes glutathione reductase (Grant *et al.*, 1996), and the additional ABC transporter proteins *PDR5* and *SNQ2* (Miyahara *et al.*, 1996).

A second AP-1-like gene, yAP-2, has also been isolated from *S.cerevisiae* (Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al.*, 1994). Although it has a similar DNA-binding specificity to yAP-1 (Wu *et al.*, 1993), it seems not to play a major function in the oxidative stress response since its deletion does not strongly affect the sensitivity to such stress (Hirata *et al.*, 1994). Furthermore, expression of some yAP-1 target genes, including *GSH1*, *TRX2* as well as a lacZ reporter gene driven by SV40 AP-1 sites (Kuge and Jones, 1994; Stephen *et al.*, 1995), is not

affected by the presence or absence of YAP2. Similarly, *YAP2* deletion had no affect on the cadmium sensitivity of the cell although its overexpression could confer some increased resistance (Wu *et al.*, 1993; Hirata *et al.*, 1994). Therefore it would appear to perform a function that, at least in some response pathways, is redundant to yAP-1. Not surprisingly, yAP-2 shows extensive homology to yAP-1 within the bZIP domain. Interestingly, however, there is a second area of extensive homology which is situated in the C-terminus (Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al.*, 1994). This region (CRD) is rich in cysteine residues and is also found in the AP-1-like protein of *Schizosaccharomyces pombe*, Pap1 (Toda *et al.*, 1991), suggesting that it may play an important functional role in the respective activities of these factors.

In this report we have addressed the mechanism of activation of yAP-1 by oxidative stress. We had shown previously that yAP-1 expression was not induced, indicating that the activation step was post-translational. Our results demonstrate that regulation is mediated by the CRD which acts to repress its transcriptional activation potential. Thus, when the CRD is removed, the resulting protein is constitutively active. Furthermore, by analysing the cellular localization of wild-type and mutant yAP-1 proteins through their fusion to green fluorescent protein (GFP), we found that removal of the CRD resulted in a striking change in localization from being predominantly cytoplasmic to being nuclear. The results suggested that the regulation of yAP-1 through the CRD region, involved regulation of nuclear localization. This was confirmed by showing that treatment of cells with diamide or diethyl maleate resulted in re-localization of wild-type protein to the nucleus. Furthermore, the CRD region is sufficient to confer oxidative stress-mediated regulation on a heterologous protein through control of its cellular localization. Three conserved cysteine residues within the CRD are important for the regulatory activity of this region.

Results

DNA-binding activity of yAP-1 is enhanced by oxidative stress

It has been shown previously that the activity of yAP-1 is enhanced by various conditions that impose oxidative stress on the cell (Kuge and Jones, 1994). A key issue concerns the mechanism of this enhancement. It is likely to result from stress-induced modification of pre-existing yAP-1 protein, since there is no evidence for increased yAP-1 expression following stress. One contributing factor could be a change in the DNA-binding activity of yAP-1, since a modest increase in binding was detected following oxidative stress imposed by diamide or diethyl maleate treatment (Kuge and Jones, 1994). In order to investigate the contribution of such a change in more detail, a yeast strain was constructed where yAP-1 expression was controlled by the constitutive promoter, cup1 cp. During the construction of this heterologous gene, we ensured that the small open reading frame (uORF) in the 5′ non-coding region of *YAP1* mRNA was removed (Figure 1A). Such a uORF is present in the 5′ untranslated region of both the *YAP1* and *YAP2* genes and it has been suggested that they could mediate regulation of *YAP1/YAP2* expression at the translational level (Bossier *et al.*, 1993).

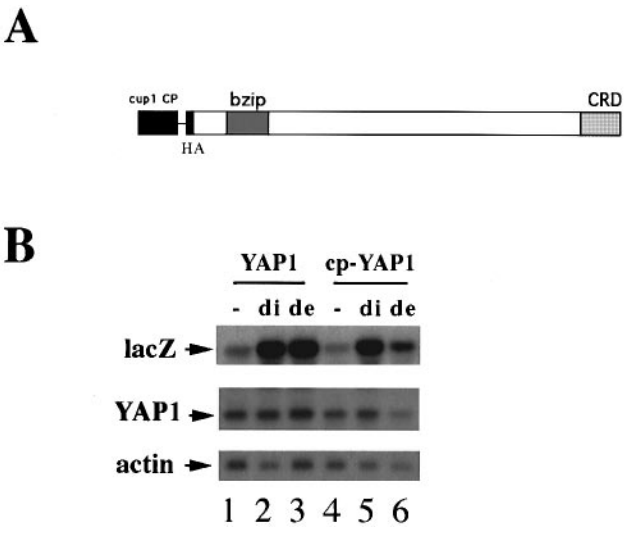

Fig. 1. Transcriptional activity of HA-tagged yAP-1 protein expressed from a constitutive promoter. (**A**) A schematic diagram of the HA-tagged YAP1 gene regulated by the cup1 cp promoter. (**B**) Northern blotting analysis of yAP-1 dependent transcription. Wild-type cells (lanes 1–3) and cp HA *YAP1* cells (lanes 4–6) were treated with 1.5 mM diamide (lanes 2 and 5) or 2 mM diethyl maleate for 90 min as described previously (Kuge and Jones, 1994). RNAs were isolated, separated and hybridized with probes that detected the indicated transcripts.

In eukaryotic cells, expression of most genes is controlled by multiple transcription factors acting co-operatively (Robertson *et al.*, 1995). This is the case for many of the identified yAP-1 target genes; *GSH1* (Stephen *et al.*, 1995), *PDR5* and *SNQ2* (Miyahara *et al.*, 1996) are all controlled by multiple factors which include yAP-1. Thus, in order to study in isolation the activity of yAP-1, we employed a lacZ reporter gene driven by SV40 AP-1 sites and the TATA element of the *cyc1* promoter; this reporter is solely regulated by yAP-1 (Kuge and Jones, 1994).

Expression of the yAP-1-dependent lacZ reporter gene was induced by diamide and diethyl maleate to a similar extent in wild-type cells and cells containing the cp-*YAP1* gene (Figure 1B). In the latter case, there was a small reduction in the diethyl maleate-induced level of lacZ but this was accompanied by slightly lower levels of *YAP1* gene expression (Figure 1B). The levels of yAP-1 protein present following diamide and diethyl maleate treatment were 0.5- and 0.6-fold higher in the cp-*YAP1*-containing cells than in untreated cells (Figure 2A). There was also a modest enhancement (1.9-fold) of yAP-1-specific DNA-binding activity in the diamide-treated extracts, whereas in the diethyl maleate-treated extracts binding activity was similar (0.9-fold) to that found in untreated cells (Figure 2B). Thus, by comparing yAP-1 protein levels to DNA-binding activity, it is clear that treatment with diamide and diethyl maleate does result in some increase in DNA-binding specific activity (3.7-fold in the case of diamide and 1.5-fold with diethyl maleate). However, since the increase is modest, and since there is significant DNA-binding activity in extracts from untreated cells (Figure 2B), it seemed unlikely that this change could fully explain the highly regulated yAP-1-dependent transcription seen upon imposition of oxidative stress.

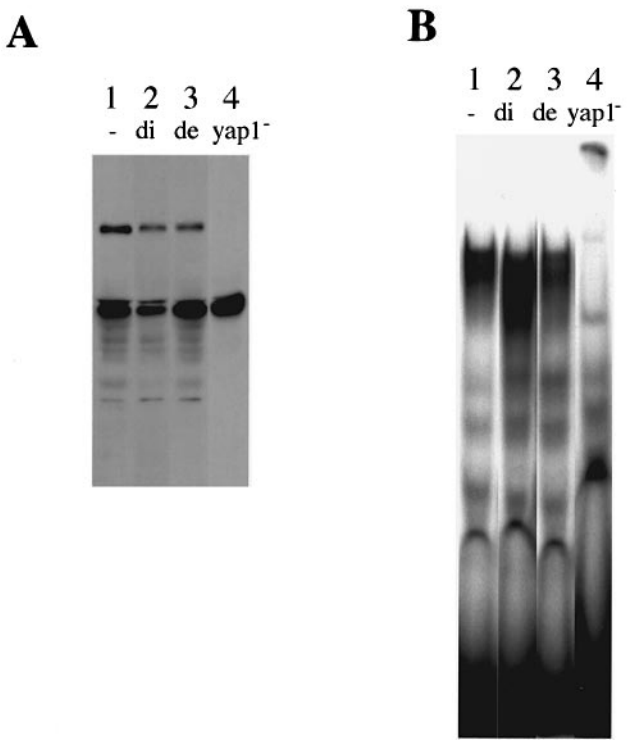

Fig. 2. yAP-1-specific DNA-binding activity is enhanced by oxidative stress caused by diamide or diethyl maleate treatment. (**A**) Western blotting and (**B**) EMSA was performed with lysates isolated from cp HA *YAP1* cells without treatment (lane 1), treated with 1.5 mM diamide (lane 2) or treated with 2 mM diethyl maleate (lane 3) as described (Kuge and Jones, 1994). A lysate from *yap1*-deficient cells (DWYU) was used as a control (lane 4). In the EMSA, the probe used was TRX2 site 2 as described previously (Kuge and Jones, 1994). The 12CA5, anti-HA monoclonal antibody was used for the Western blotting.

The carboxy-terminal region of yAP-1 is essential for regulation by oxidative stress

The results described above suggested additional levels of post-translational control. In order to investigate the nature of such controls, the functional importance of different yAP-1 domains was addressed. The genomic *YAP1* gene was replaced with various deletion mutants that removed one or more previously recognized domains, namely the cysteine-rich domain (CRD) at the C-terminus (Toda *et al.*, 1991) and the two transcriptional activation domains (Wemmie *et al.*, 1994b) located between the CRD and bZIP region (Figure 3A). Thus, in the *yap1* (1-571) mutant, the CRD has been removed whereas in *yap1* (1-373) both the CRD and the activation domain II are missing. The mutant *yap1* (1-244) lacks all three domains.

When expression of a yAP-1-dependent lacZ reporter gene was examined in the various mutant strains, we were surprised to find that both the mutants *yap1* (1-571) and *yap1* (1-373) showed high levels of basal activity; ~10-fold more lacZ expression occurred in *yap1* (1-373) -expressing cells than in wild-type cells in the absence of inducing conditions and upon diamide or diethyl maleate treatment there was an additional 2-fold increase (Figure 3B and C). These results suggest that the CRD region acts as a negative regulator of transcriptional activation and that induction by oxidative stress entails removal of this negative regulation rather than stimulation of the activation process. *yap1* (1-244) showed no activity in either induced or uninduced conditions, consistent with the removal of both the previously described transcription activation domains (Wemmie *et al.*, 1994b).

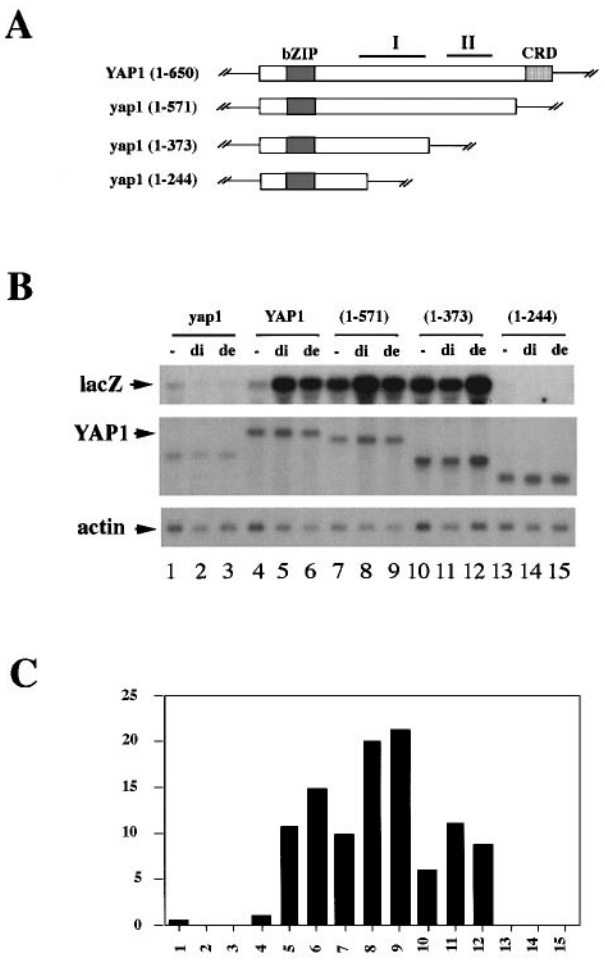

Fig. 3. Transcriptional activity of deletion mutants of yAP-1 protein. (**A**) Schematic representation of the deleted regions of the *YAP1* genes integrated into the *YAP1* locus. The C-terminal cysteine-rich region (CRD) and basic leucine zipper region (bZIP) are indicated as are the two previously described transcriptional activation domains (I and II) (Wemmie *et al.*, 1994b). (**B**) *yap1* disruption mutant (DWYU) (lanes 1–3), wild-type (DY) (lanes 4–6), *yap1* (1-571) (lanes 7–9), *yap1* (1-373) (lanes 10–12) and *yap1* (1-244) (lanes 13–15) cells were treated with diamide (lanes 2, 5, 8, 11 and 14) or with diethyl maleate (lanes 3, 6, 9, 12 and 15) or left untreated (lanes 1, 4, 7, 10 and 13). RNAs were isolated and analysed for specific transcripts as indicated. (**C**) Expression level of the lacZ reporter gene. The level of lacZ RNA was quantified by scanning and analysed by NIH image. The level of lacZ RNA was normalized to the level of actin RNA.

It was important to compare the expression of each of the mutant proteins. At the RNA level, no difference was seen between the *yap1* mutants and the wild-type *YAP1* gene (Figure 3B). At the protein level however, the picture was significantly different (Figure 4). The level of the constitutively active yAP-1 (1-571) protein was greatly reduced. Levels of yAP-1 (1-373) and yAP-1 (1-244) proteins were also lower than in the wild-type case, but the reductions were modest and fairly insignificant compared with yAP-1 (1-571). The results demonstrate that the CRD region has a role in determining the stability of the yAP-1 protein, its removal resulting in increased protein turnover. This is consistent with the results of Wemmie *et al.* (1994b), who also showed that the removal

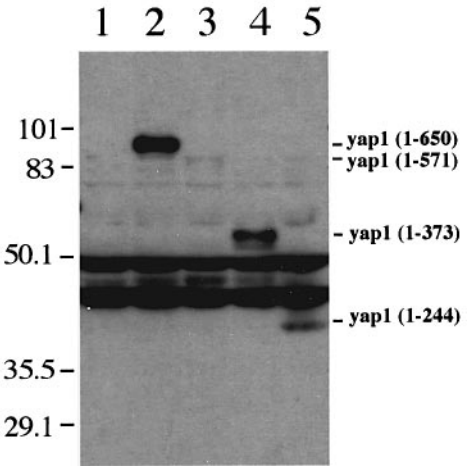

Fig. 4. Western blotting analysis of yAP-1 mutant proteins. Lysates were prepared from the *yap1* disruption mutant (DWYU) (lane 1), DWYU carrying pRS cp-HA YAP1 (lane 2), pRS cp-HA *yap1* (1-571) (lane 3), pRS cp-HA *yap1* (1-373) (lane 4) or pRS cp-HA *yap1* (1-244) (lane 5). Immunoblotting was performed with anti-yAP-1 antibody.

of the C-terminal region led to a decrease in yAP-1 levels as judged by DNA affinity blotting. The observation that the steady-state levels of yAP-1 (1-373) were significantly higher than yAP-1 (1-571) suggests that the turnover of the yAP-1 protein may depend upon sequences located between residues 374 to 571. For example, if yAP-1 turnover is ubiquitination-dependent, then this region may be necessary for interaction with the ubiquitination machinery. Despite the low levels of the yAP-1 (1-571) protein, it was sufficient to fully activate the lacZ reporter gene (Figure 3B and C).

Wild-type yAP-1 is predominantly cytoplasmic whereas the constitutively active yAP-1 mutants are localized to the nucleus

Since the activity of yAP-1 does not correlate with protein levels, we considered the possibility that activity might be regulated at the level of nuclear localization. To this end, we employed (GFP) Green Fluorescent Protein (Chalfie *et al.*, 1994) to detect the yAP-1 protein in live yeast cells. Full-length and deletion mutants of yAP-1 were fused at their N-termini to the GFP coding region; expression of the hybrid genes was controlled by the cup1 cp promoter (Figure 5A). As shown in Figure 6A, the GFP–yAP-1 fusion supported diamide- and diethyl maleate-induced transcription of the yAP-1-dependent lacZ reporter gene; thus, its regulation was indistinguishable from the normal, unfused protein.

The GFP–yAP-1 protein was detected throughout the cells (Figure 5B, panel 1). In contrast, however, both the GFP–yAP-1 (1-571) and GFP–yAP-1 (1-373) proteins were concentrated into single spots (Figure 5B, panels 2 and 3). This was also the case for the transcriptionally inactive mutant GFP–yAP-1 (1-244) (Figure 5C, panel 4) and a comparison with DNA staining (Figure 5C, panels 5 and 6 and data not shown) clearly showed that the protein was concentrated in the nuclei of the cells. These results indicated that the CRD regulates the localization of the yAP-1 protein, its removal allowing efficient accumulation in the nucleus. Since removal of the CRD also results in constitutive transcriptional activity, they further suggest that regulation of yAP-1 nuclear import mediated by the CRD region, could represent a major underlying mechanism of yAP-1 induction.

Induction of yAP-1 by oxidative stress correlates with its nuclear import

We next tested whether the localization of GFP–yAP-1 was modulated by oxidative stress imposed by diamide and diethyl maleate treatment. In both cases, treatment resulted in concentration of the GFP–yAP-1 protein consistent with the induction of nuclear localization (Figure 6B, compare panel 1 with panels 2 or 3). In order to verify that the protein was indeed nuclear, the GFP fluorescence pattern of diethyl maleate-treated cells was directly compared with staining of nuclei by propidium iodide. As shown in Figure 6C, the signals were coincident.

The yAP-1 CRD can confer regulated localization on a heterologous transcription factor

The CRD region is essential for the regulated localization of the yAP-1 protein. In order to address whether it is sufficient and whether it can confer such regulation on a heterologous protein, we constructed a hybrid containing the GAL4 DNA-binding domain (GAL4 dbd) fused to GFP and the yAP-1 CRD (Figure 7A). The behaviour of this fusion following oxidative stress was examined. We found that the localization of this fusion was regulated; in untreated conditions it was dispersed throughout the cells (Figure 7B, panel 1), but became concentrated when the cells were exposed to oxidative stress conditions (Figure 7B, panels 2 and 3). Again, by comparing GFP fluorescence and nuclear staining, it was clear that this concentration represented nuclear localization (Figure 7B, panels 4–6). The results indicated that the CRD was sufficient to confer regulated nuclear localization of a heterologous protein.

Conserved cysteine residues in the CRD are essential for regulation

The CRD region of yAP-1 is highly conserved with the C-terminal regions of the yAP-2 factor and the fission yeast factor, Pap1 (Figure 8A) (Moye-Rowley *et al.*, 1989; Hussain and Lenard, 1991; Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al.*, 1994). Of particular note are the three cysteine residues at positions 598, 620 and 629 which are absolutely conserved in all three cases. Since a specific cysteine residue has been shown to be essential for the redox sensitivity of the bacterial OxyR protein (Kullik *et al.*, 1995), we examined the effect of amino acid substitutions at each of the cysteine residues in the CRD. All three were individually substituted to threonine, which has a molecular size similar to cysteine (Figure 8A). As shown in Figure 8B, GFP-fused versions of yAP-1 C598T and yAP-1 C629T, behaved like the wild-type yAP-1 protein (Figure 8B, panels 1 and 3), showing a diffused localization pattern. In contrast, however, the yAP-1 C620T mutant protein localized to the nucleus (Figure 8B, panels 2 and 6–9). These results were consistent with the transcriptional activation of the reporter lacZ gene by these mutant yAP-1 proteins. In C598T- and C620T-expressing cells, the regulation of yAP-1-dependent lacZ transcription was essentially wild-type in nature (Figure

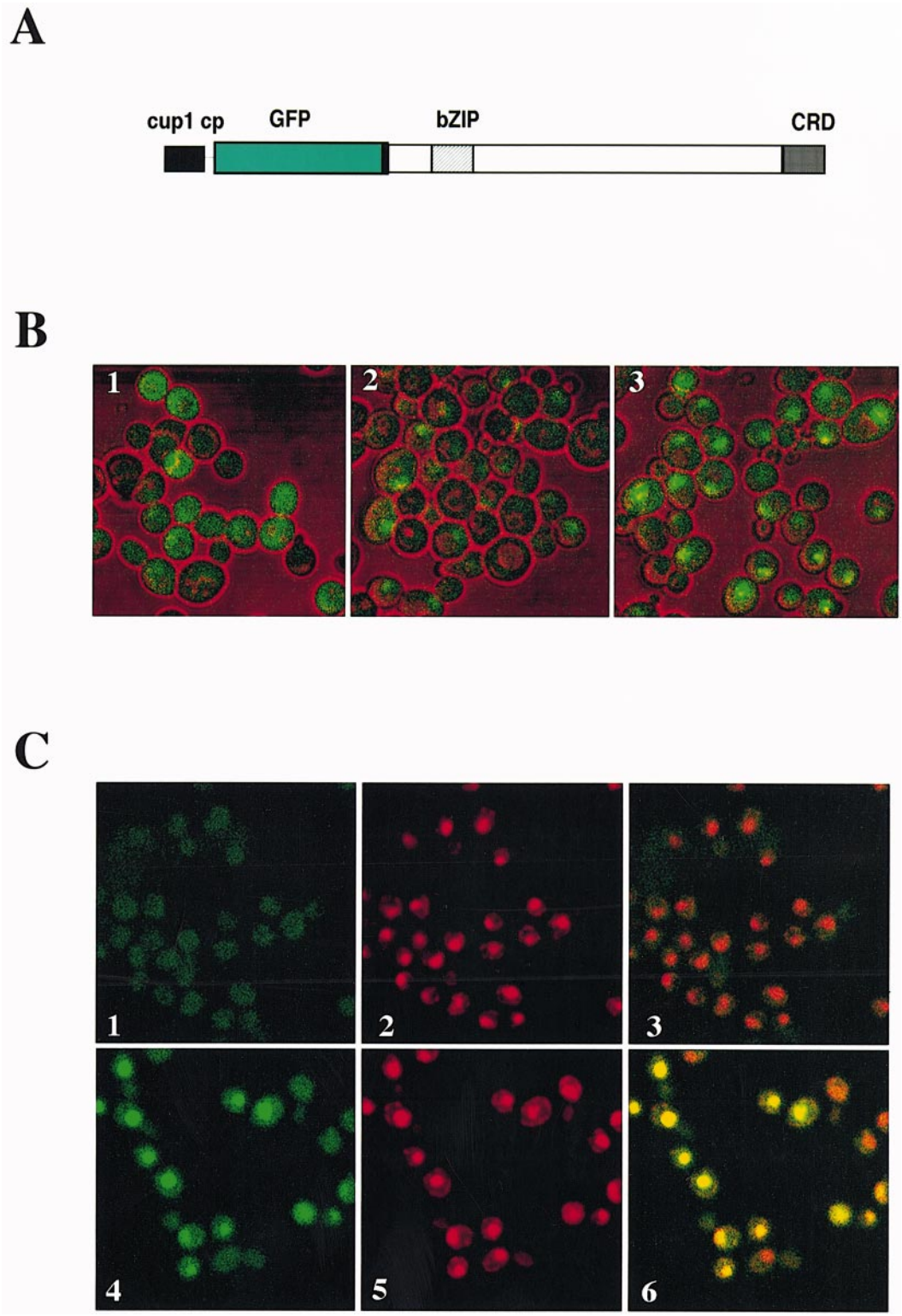

Fig. 5. (**A**) A schematic of the GFP–*YAP1* fusion gene. (**B**) Confocal laser scanning microscopic analysis of the cellular distribution of GFP-tagged yAP-1 wild-type and mutant proteins. *yap1* disruption mutant (DWYU) cells carrying pRS cp-GFP–YAP1 (panel 1), pRS cp-GFP–*yap1* (1-571) (panel 2) or pRS cp-GFP–*yap1* (1-373) (panel 3) were analysed. Images of GFP (green) and transmitted (red) light were merged. (**C**) Localization of *yap1* deletion mutant proteins. Fluoresence analysis and propidium iodide staining of DWYU (panels 1–3) or DWYU cells carrying pRS cup cp-GFP–*yap1* (1-244) (panels 4–6) were carried out as described in Materials and methods. Confocal laser scanning microscopic background fluorescence (panel 1) or GFP fluorescence (green, panel 4), DNA stained with propidium iodide (red, panels 2 and 5) and the GFP or background fluorescence merged with the propidium iodide (PI) staining (panels 3 and 6).

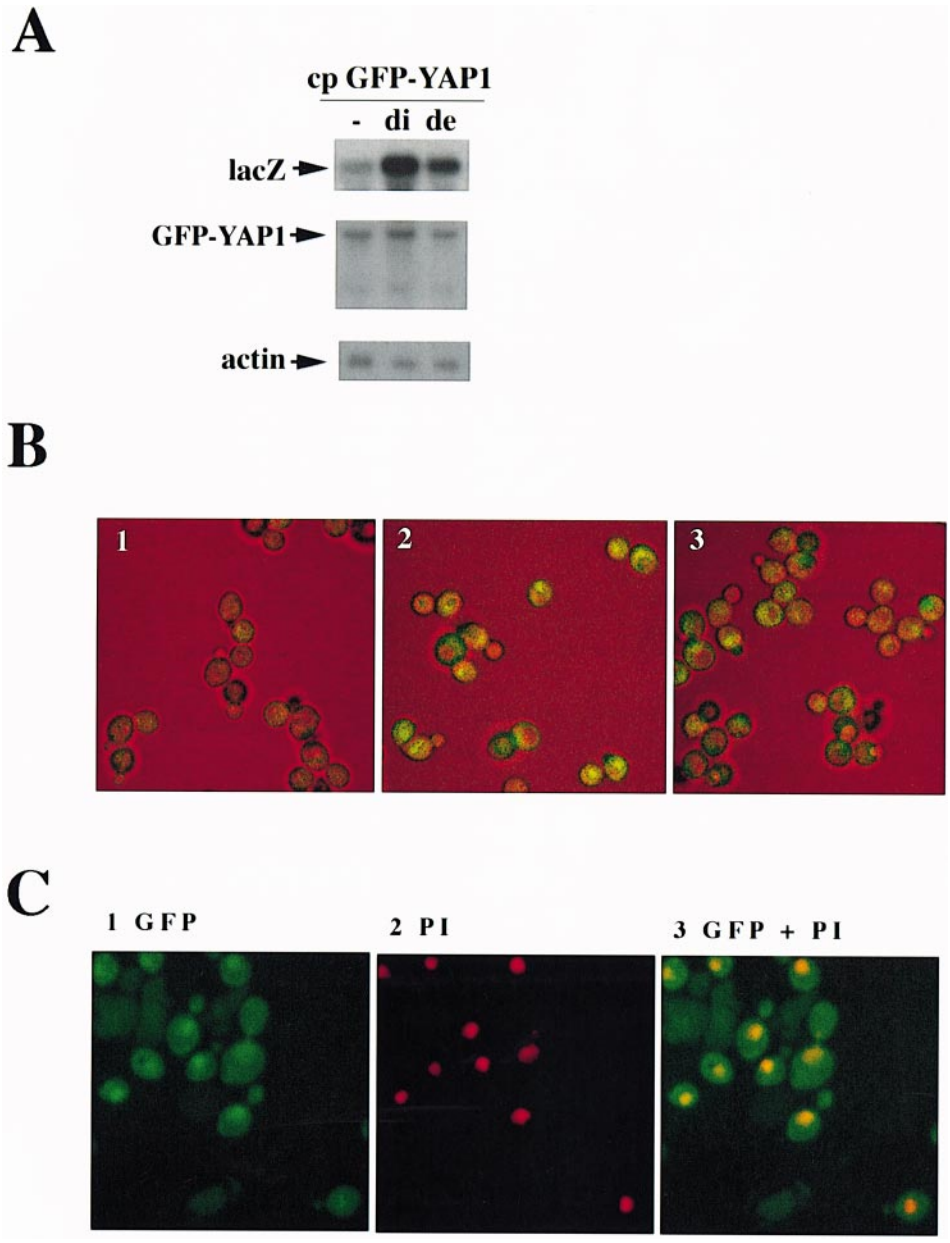

Fig. 6. Oxidative stress enhances the nuclear import of the yAP-1 protein. (**A**) Northern blotting analysis of cp-GFP–*YAP1* cells. The cp-GFP–*YAP1*-expressing cells were treated with diamide (di), diethyl maleate (de) or left without treatment (–). Northern blotting analysis was carried as described in Figure 1. (**B**) Confocal laser scanning microscopic analysis of cup1 cp-GFP–*YAP1* cells without treatment (panel 1), and following treatment with diamide (panel 2) or diethyl maleate (panel 3). Transmitted light (red) and GFP fluorescence (green) were merged. (**C**) Co-localization of GFP fluorescence and PI DNA staining. The GFP fluorescence (panel 1) and PI staining (panel 2) patterns of diethyl maleate-treated cp-GFP–*YAP1* cells were merged (panel 3).

9A). However, in the case of C620T cells, regulation was lost and expression was predominantly constitutive (Figure 9A and B, lanes 3 and 4). In addition, a double mutant with threonine substituted at positions C598 and C629 (cm46) was constructed. This mutant had a wild-type pattern of behaviour (Figure 8B, panel 4 and Figure 9A). It appeared therefore that, of the three cysteine residues, only the C620 residue could not be substituted to threonine without loss of CRD function. Fusion of the GAL4 dbd–GFP protein with the CRD containing the C620T mutation also resulted in constitutive nuclear localization (Figure 8B, panels 5 and 10–13), supporting our conclusion that this residue is critical for CRD function.

To examine how other amino acid substitutions at position C620 would affect the localization of yAP-1, we substituted C620 with the negatively charged residue, aspartic acid or the hydrophobic residue, alanine (Figure 8A). The cellular localization of these mutant proteins is shown in Figure 8C. The C620D mutant behaved identically to the C620T mutant; in other words, localization was predominantly nuclear (Figure 8C, panels 1–4). In contrast, C620A was wild-type in character with the protein being predominately cytoplasmic (Figure 8C, panels 5–9). This behaviour was consistent with their ability to regulate the expression of the yAP-1-dependent lacZ reporter gene; GFP–C620D-expressing cells showed

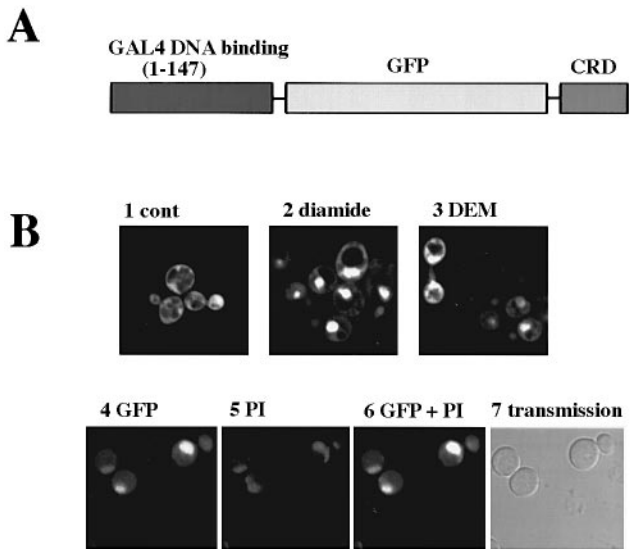

Fig. 7. The CRD confers regulated nuclear localization on the GAL4 DNA-binding domain in response to oxidative stress. (**A**) Schematic of the GAL4 dbd–GFP-CRD fusion protein. (**B**) Confocal laser scanning microscopic analysis of the GFP fluorescence in Y187 cells (Clontech Laboratories) expressing the GAL4 dbd–GFP-CRD fusion without treatment (panel 1), and following treatment with diamide (panels 2 and 4) or diethyl maleate (panel 3) is shown. The diamide-treated cells were fixed and stained with PI (panel 5) and the PI and GFP fluorescence patterns merged (panel 6). A picture of the transmitted image is also shown (panel 7).

constitutive expression (Figure 9B, lanes 9 and 10) whereas in GFP–C620A-containing cells expression was induced by oxidative stress (Figure 9B, lanes 5 and 6). A slightly higher basal level of activity was seen in cells expressing the GFP–C620A protein compared to wild-type cells, which might suggest some slight deregulation. However, this deregulation was minor compared to that of the C620T and C620D mutants.

The results with the C620A mutant presented a paradox. Other substitutions at this position clearly indicated that this cysteine was important for regulation, and yet substitution with alanine had a relatively minor effect. We reasoned that, in the case of the C620A mutant, one or both of the other cysteine residues might be critical. In order to test this possibility, we substituted the C598 and C629 residues with threonine in the C620A background (cm46A5). Interestingly, localization of this mutant protein was predominately cytoplasmic and was not affected by oxidative stress (Figure 8 C, panels 10 and 11). This result was consistent with its ability to activate the lacZ reporter gene in response to oxidative stress. The basal activity of GFP–cm46A5 was similar to GFP–C620A; however, GFP–cm46A5 could not activated by diamide (see Figure 9B, compare lanes 5 and 6 with lanes 7 and 8).

The drug-resistant phenotype of cells expressing the yAP-1 mutants

Since overexpression of the yAP-1 protein can confer drug resistance, cells expressing mutants that show constitutive nuclear localization and transcriptional activation might show increased resistance to drugs when compared with wild-type cells. This possibility was examined with the yAP-1 mutants described above. In general, the sensitivity of mutant expressing cells to diamide and diethyl maleate was consistent with their regulatory properties. For example, cells expressing the constitutively active yAP-1 mutants *yap1* (1-571) and C620T were found to be partially resistant to 1.5 mM diamide and 2 mM diethyl maleate, conditions that were toxic for wild-type cells (see Table I). However, in terms of H_2O_2 sensitivity, the picture was complex. The resistance phenotype of the C620T, C598T and C629T mutant-expressing cells, as well as wild-type cells, was identical showing that the constitutive nature of C620T did not translate into a resistance phenotype. Surprisingly, however, the CRD does seem to be important for such resistance, since the yAP-1 (1-571) mutant was found to be hypersensitive and failed to grow in the presence of 1 mM H_2O_2.

Discussion

The yAP-1 transcription factor is crucial for the normal response of cells to a variety of stress conditions including oxidative stress, stress mediated by many drugs, and heat shock. Importantly, all of these conditions induce activity of yAP-1 and, as a consequence, result in increased expression of a diverse range of genes encoding proteins that help protect the cell against stress-induced damage (Kuge and Jones, 1994; Stephen *et al.*, 1995; Miyahara and Hirata, 1996). A major, unresolved question concerns the mechanism of this induction. Based on the results reported here, we suggest that the regulation of yAP-1 activity by oxidative stress occurs at two levels. There is a modest increase in the DNA-binding ability of yAP-1, but the predominant mode of regulation is at the level of its cellular localization and that upon induction, yAP-1 is selectively re-localized from the cytoplasm to the nucleus. This conclusion is supported by two main lines of evidence. Firstly, constitutively active forms of yAP-1 are localized to the nucleus, whereas the wild-type protein is predominantly cytoplasmic. Secondly, upon induction with diamide or diethyl maleate, wild-type yAP-1 protein translocates from the cytoplasm to the nucleus.

yAP-1 joins a growing list of important transcription factors that are regulated at the level of nuclear import (Vandromme *et al.*, 1996). The list includes other yeast factors such as SWI5, whose localization is regulated in a cell cycle-dependent manner (Moll *et al.*, 1991) and the mammalian factor NF-κB which relocates, from the cytoplasm to the nucleus in response to a variety of mitogens, cytokines or stress-induced signals (Blanck *et al.*, 1992). An important question concerns the mechanism of regulated yAP-1 nuclear localization. At least two models can be envisaged. In one model, the regulation could be due to a post-translational modification that affects the availability of a nuclear localization signal (NLS), as is the case for the regulated localization of the SWI5 protein (Moll *et al.*, 1991). In the other model, modification could affect interaction with a cellular protein which serves to sequester yAP-1 to the cytoplasmic compartment. Which of these two mechanisms, if either, might be operating is unknown at present, although the former model is probably unlikely for two reasons. First, although the NLS of yAP-1 has not been mapped, it is likely to be N-terminal since the mutant *yap1* (1-244) localizes to the nucleus. It is thus well separated from the CRD. Second, the CRD can impose regulated nuclear

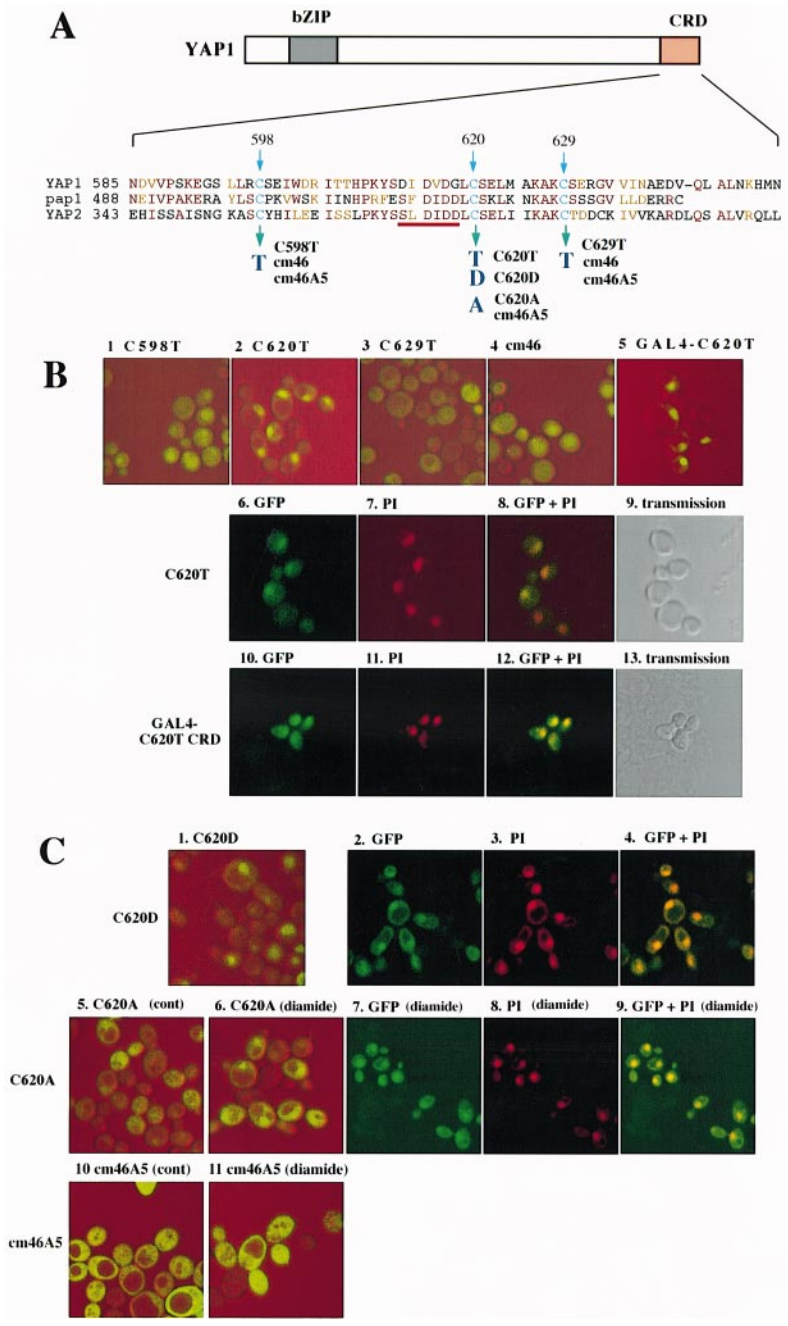

Fig. 8. The involvement of specific cysteine residues in CRD function. (**A**) Alignment of the CRD regions of yAP-1, yAP-2 (Moye-Rowley *et al.*, 1989; Hussain and Lenard, 1991; Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al.*, 1994) and pap1 (Toda *et al.*, 1991). More than two out of three identical amino acid residues are shown in red and structurally similar amino acid residues are shown in yellow. The consensus cysteine residues are shown in blue. The amino acids substituted in various mutants are indicated below the sequence. The acidic stretch of residues flanking C620 is indicated with the red bar. (**B**) Localization of mutant yAP-1 proteins containing Cys → Thr substitutions. The GFP fluorescent (green) and the transmitted images (red) were merged (panels 1–5). Shown are the fluorescence images of C598T (panel 1), C620T (panel 2), C629T (panel 3), cm46 (panel 4) and the GAL4 dbd-GFP fused with the CRD of C620T (GAL4–C620T CRD) (panel 5). Cells expressing the C620T and GAL4–C620T CRD proteins were also stained with PI. The GFP fluorescence (panels 6 and 10) and PI fluorescence (panels 7 and 11) were merged (panels 8 and 12). Transmitted images (panels 9 and 13) are also shown. (**C**) Localization of the C620D, C620A and cm46A5 proteins. The GFP fluorescent (green) and the transmitted images (red) were merged (panels 1, 5, 6, 10 and 11). Shown are the fluorescence images of C620D (panel 1), C620A (panel 5) and cm46A5 (panel 10) -expressing cells. In addition the GFP (panel 2) and PI (panel 3) fluorescence patterns of C620D were compared (panel 4). The effect of diamide treatment on the localization of the C620A (panels 6–9) and cm46A5 (panel 11) mutant proteins was also analysed. The GFP fluorescence (panel 7), PI fluorescence (panel 8) and merged patterns (panel 9) of diamide-treated C620A expressing cells are shown.

localization on a heterologous protein, showing that the exact nature and position of the NLS is unimportant. We thus favour the model which evokes an inhibitor protein which we are currently trying to identify.

A major question that arises from this work is the question of the mechanism that operates to regulate yAP-1 localization by oxidative stress. One possibility is that the imposition of such stress activates a signalling pathway that results in the phosphorylation and modulation of either yAP-1 or a putative inhibitor protein. An alternative

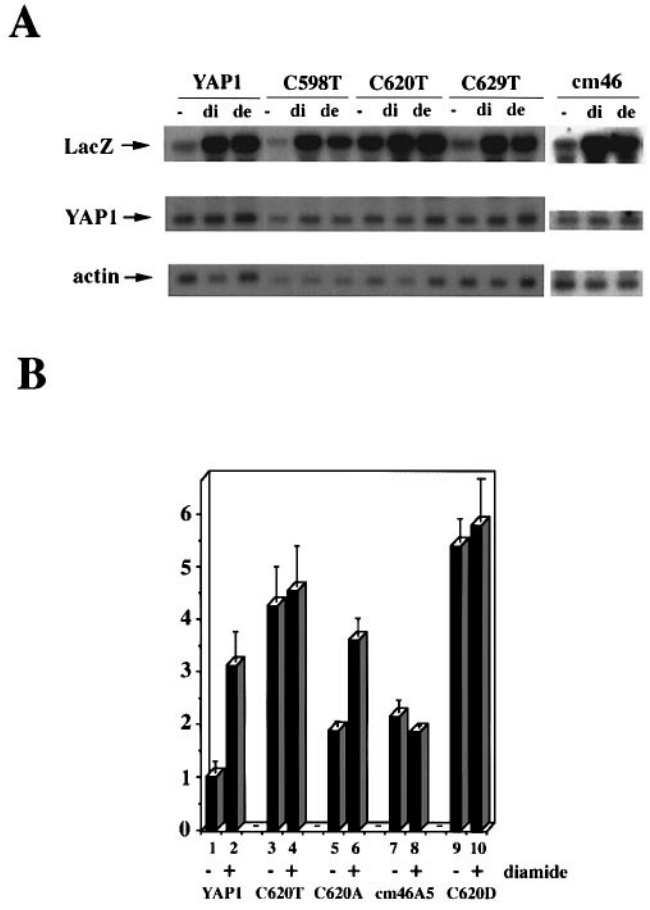

Fig. 9. (**A**) The transcriptional activity of Cys-substitution mutants was tested. The genomic YAP1 gene was replaced with the GFP fused C598T, C620T, C629T or cm46 mutant genes. Northern blotting analysis of RNA isolated from such cells without treatment or after diamide (di) or diethyl maleate (de) treatment was carried out as described in the legend to Figure 3. (**B**) Transcriptional activity of mutant yAP-1 proteins C620T, C620A, cm46A5 and C620D. β-Galactosidase activity was assayed as described previously (Kuge and Jones, 1994) in DWYU cells expressing GFP protein fused with the yAP-1 (1 and 2), C620T (3 and 4), C620A (5 and 6), cm46A5 (7 and 8) and C620D (9 and 10) proteins. Cells were either untreated (1, 3, 5, 7 and 9) or treated with diamide (2, 4, 6, 8 and 10) as described in Figure 1. The relative β-galactosidase activities are indicated. The background levels of β-galactosidase in *yap1*$^{-}$ cells treated identically were deleted from the values obtained. The results represent the values from three independent cultures.

possibility is that the redox state of the cell modulates the activity of yAP-1 or possible interacting proteins in a more direct fashion by the oxidation of critical amino acid residues. This latter possibility is supported by our initial mutational analysis of the CRD region. It is clear from this analysis that the three conserved cysteine residues are important, and in particular the residue at position 620. It is tempting to speculate therefore, that the oxidation of -SH groups of the cysteine residues alters the behaviour of the yAP-1 protein, either by inducing a conformational change or by decreasing the hydrophobicity of the CRD region. The C620 residue might be particularly significant with respect to the hydrophobic nature of the CRD, since it is flanked by acidic residues which increases the p*K* of the -SH groups (Lindley, 1960, 1962). This could explain the different consequences of alternative substitutions at this position. A change to threonine or aspartate would increase hydrophilicity at this position and hence would be expected to have a more deleterious effect than a change to the more neutral alanine residue. In the context of our favoured model involving a cytoplasmic inhibitor protein, a redox-sensitive change in the conformation or hydrophobicity of the CRD could result in loss of protein–protein interaction, thus releasing yAP-1 for transport into the nucleus.

As well as changing the cellular localization of yAP-1, inducing conditions also result in a modest increase in its DNA-binding capacity. This enhancement can be seen not only with the wild-type protein, but also with the mutants that we have examined in this study, including yAP-1 (1-244) (data not shown). These results suggest the possibility of an unknown post-translational modification induced by oxidative stress occurring within the N-terminal DNA-binding domain. In addition, this enhancement of DNA-binding activity by oxidative stress would explain the modest activation of yAP-1-dependent transcription by the nuclear localized yAP-1 (1-571) and yAP-1 (1-373) mutant proteins.

Cells expressing mutants that show constitutive nuclear localization [yAP-1 (1-571) and C620T] had slightly increased expression levels of GSH1 and GLR1 (data not shown). This could explain their increased resistance phenotype to thiol oxidants such as diamide and diethyl maleate. However, this was not the case with the *TRX2* gene, which was regulated by thiol oxidants and H_2O_2 normally. These results suggest that yAP-1 is not the only

Table I. Drug resistance of yAP-1 mutants[a]

	None	Diamide		Diethyl maleate		H_2O_2	
		1 mM	1.5 mM	1 mM	2 mM	0.5 mM	1 mM
YAP1 (W303B)	++	++	−	+	−	++	+
yap1- (DWYU)	++	−	−	−	−	−	−
yap1 (1-571)	++	++	+	++	+	++	−
yap1 (1-373)	++	+	−	++	−	+	−
C598T	++	+	−	++	−	++	+
C620T	++	++	+	++	+	++	+
C629T	++	++	−	++	−	++	+
cm46	++	+	−	++	−	+	−

[a]The drug resistance phenotype was determined as follows. The indicated strains were cultured in liquid YPAD medium to log phase. 2 μl of the culture were then spread onto YPAD agar plates containing the indicated drugs. The growth of the cells was monitored after incubation for 50 h at 30°C. ++, wild-type growth; +, partially inhibited growth; −, no growth observed.

factor that is important for *TRX2* regulation by oxidative stress; therefore, even though yAP-1 is deregulated in the mutant-expressing cells, expression cannot take place until the additional factor is activated. This behaviour of *TRX2* might explain why cells expressing the constitutive mutants do not show greater resistance to H_2O_2.

Materials and methods

Yeast strains, media, enzymes and reagents

The wild-type and Δyap1 cells carrying a lacZ reporter gene driven by SV40AP-1 binding sites were as follows; wild-type, DY [*MAT α his3 can1-100 ade2 leu2 trp1 ura3 ura3*::(3xSV40AP1-lacZ)] and *yap1* disruption mutant, DWYU [*MAT α his3 can1-100 ade2 leu2 trp1 yap1::URA3 ura3*::(3xSV40AP1-lacZ)] (Kuge and Jones, 1994). The trp drop-out media and yeast minimal medium supplemented with amino acids was as described by Kuge and Jones (1994). Restriction enzymes were purchased from New England BioLabs, Ex Taq polymerase and other DNA-modifying enzymes were from Takara Syuzo Co. Diamide, diethyl maleate and thrombin were purchased from Sigma.

Construction of yeast strains containing modified yap1 genes

Before generating *yap1* deletion mutants, the following double-stranded oligonucleotide-containing translation termination codons in each frame was inserted into the *Nde*I site (nucleotide number from initiation codon 1942/1943) of pUC-YAP1(S-E) (Kuge and Jones, 1994), at the 3′ end of the coding region:

5′-TATGAACTAACTAACTAA-3′
3′-ACTTGATTGATTGATTAT-5′

The resulting plasmid DNA designated pUC–YAP1-3, was digested with *Nde*I and treated with *Bal*31 exonuclease. After digestion with *Bam*HI, the fragments with various deletions in the YAP1 coding region were isolated from an agarose gel and recloned into the *Bam*HI and *Nde*I sites of pUC–YAP1-3. To make yAP-1 (1-571), pUC–YAP1-3 was digested with *Nde*I and *Bsm*I, blunt-ended and then ligated. These mutant plasmids were digested with *Sph*I and *Eco*RI and introduced into DWYU cells in which the bZIP domain of YAP1 was replaced with the *URA3* gene. After isolation of ura$^-$ colonies by 5-FOA selection, individual colonies were analysed by Southern blotting and PCR (primer chain reaction) to confirm that gene replacement had take place.

Construction of GFP-tagged and HA-tagged yap1 genes

pGFP-RV (provided by Dr Y.Watanabe, Teikyo University) contains the GFP coding region cloned into the *Eco*RV site of pBluescript. To make the S65T mutant of GFP (Heim *et al.*, 1995), PCR was carried out using the following primers: 5′-TGTTCCATGGCCAACACTTGTCACTACT-TTCACTTATGGTG-3′ and M13-47 primer (Takara shuzo Co.), which can prime at the lacZ coding region next to the polylinker. After digestion with *Nco*I and *Xho*I, this fragment was replaced into the same plasmid to generate pGFP(S65T). Since a moderately constitutive active promoter was required for expression of the GFP-fusions, a truncated cup1 promoter (–59 to –246 from the initiation codon; cup1 cp) was used. The cup1 promoter and the GFP(S65T) coding region were cloned into pRS314 (TRP1, CEN6-ARSH4) (Shikorski and Hieter, 1989) to generate pRS-cup-GFP. Using the following the two oligonucleotides: 5′-GTCA-GAGCTCTCTTTTGCTGGCATTTCTTCTAGA-3′ and 5′-ACTGCA-GCTGACTTGTATAGTTCATCCATGCC-3′.

The cup1 cp-GFP region was PCR amplified and digested with *Sac*I and *Pvu*II. A fragment containing most of the coding region (corresponding to amino acids 4 to 650) of YAP1 was isolated, by digestion with *Acc*I and *Eco*RI. These cup-GFP and YAP1 DNA fragments were cloned between the *Sac*I and *Eco*RI sites of pRS314 and the resulting plasmid was designated pRS cup1 cp-GFP–YAP1. As a result of this construction, the C-terminal 12 amino acid sequence of GFP was deleted and amino acid position 226 of GFP was fused to amino acid position 5 of yAP-1. To make GFP-tagged *yap1* deletion mutants, YAP1 wild-type sequence of pRS cp-GFP–YAP1 were replaced by corresponding regions of these various mutant sequences following digestion with *Bam*HI (nucleotide position 185/186) and *Bst*EII (nucleotide position 1976/1975). To generate HA-tagged versions of *YAP1* or GFP–*YAP1*, the *Pvu*II–*Bam*HI fragment from the 5′ end of the sequences was replaced by a YAP1 fragment generated by PCR using the following oligonucleotides: 5′-GTCACAGCTGCCATGTACCCATACGATGTTC-CAGATTACGCTAGTGTGTCTACCGCCAAGAGG-3′ and 5′-GGCG-AAAAGGCGAAGCAAGGT-3′. The resulting plasmids were designated pRS cp-GFP HA YAP1 and pRS cp-HA YAP.

Construction of yeast strains containing cup1 cp-YAP1

A fragment containing the upstream region of the YAP1 gene, from *Sph*I (–608) to *Dra*I (–27), was ligated upstream of the cup1 promoter of pRS cp-GFP HA YAP1 or pRS cp-HA YAP1 and transfected into DWYU to replace to *yap1::URA3* as described above.

Confocal laser scanning microscopic analysis

Live yeast cells were observed without fixing by Bio-Rad MRC1024 installed with a Kr/Ar laser and Nikon Optiphot 2 microscope with a Nikon Plan Apo 60 1.4 objective. Scanning was performed by 488 nm laser (GFP) or 568 nm laser (PI) and the fluorescence was detected using the T1/T2A filter set. For the DNA staining experiment, cells were fixed with 4% paraformaldehyde for 3 h. After washing twice with 0.2 M Tris–HCl, pH 7.5, cells were treated with 1 mg/ml of RNase A at 37°C for 3 h and stained with 5 μg/ml propidium iodide for 15 min.

Construction of a GAL4 bd-GFP–yAP-1 CRD fusion gene

A plasmid vector pAS2 (Clontech Laboratories) containing the GAL4 DNA-binding domain (amino acids 1–147) was used. The CRD (578–650) was isolated by PCR and cloned between the *Nco*I and *Sal*I sites of this vector. Subsequently, the GFP S65T coding region flanked by *Bsp*HI sites was isolated by PCR and cloned into a *Bsp*HI site located between the GAL4 bd and the CRD.

Substitution of the cysteine residues of the CRD

A PCR-based strategy was employed to introduce nucleotide substitutions (Kuge and Jones, 1994) at defined locations. The codons TGT (C598, C620 and C629) were changed to ACT (T; Thr) to make *yap1* C595T, C620T, C629T and cm46. The codon TGT (C620) was changed to GCT (A; Ala) or GAT (D; Asp) to make *yap1* C620A or C620D. The mutant *yap1* genes were fused to GFP as described above and some of the resulting mutants introduced into the genomic *yap1* locus.

Protein analysis and yeast lysate preparation

Whole-cell yeast lysates were either prepared as described previously (Kuge and Jones, 1994) or for the EMSA and Western blotting experiments shown in Figure 2, by spheroplasting and homogenizing (Dunn and Wobbe, 1996). To prepare anti-yAP-1 rabbit serum, N-terminal yAP-1 sequences (5-63) were fused to GST protein and expressed in *E.coli*. The fusion protein was isolated by binding to GSH–Sepharose and digested with Thrombin. The resulting supernatant containing the yAP-1 (5-63) peptide was used to immunize New Zealand White rabbits. Antiserum was purified using the same yAP-1 peptide as described by Sambrook *et al.* (1989). Yeast protein extracts were separated by 10% SDS–PAGE and transferred onto PVDF membrane (Immobilon; Millipore) and immunoblotted using the anti-yAP-1 rabbit serum or 12CA5, anti-HA monoclonal antibody (Boehringer). To detect a specific signal, peroxidase-conjugated anti-rabbit Ig or anti-mouse Ig second antibodies (Dako) were used and detected by the ECL system (Amersham Life Science).

RNA analysis and EMSA

Northern blotting analysis and electrophoresis mobility shift assays were performed as described previously (Kuge and Jones, 1994).

Acknowledgements

We thank Takashi Toda and Mark Toone (ICRF) for their valuable advice and guidance in preparing this manuscript. We thank Yuichiro Watanabe (Teikyo University) for generously providing the GFP gene. We thank Peter Jordan (ICRF) and Sumiko Funato (Nippon Bio-Rad Laboratories) for technical assistance with the confocal microscopy. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Received on August 14, 1996; revised on December 4, 1996