H₂O₂ sensing through oxidation of the Yap1 transcription factor

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The yeast transcription factor Yap1 activates expression of antioxidant genes in response to oxidative stress. Yap1 regulation involves nuclear accumulation, but the mechanism sensing the oxidative stress signal remains unknown. We provide biochemical and genetic evidence that upon H₂O₂ treatment, Yap1 is activated by oxidation and deactivated by enzymatic reduction with Yap1-controlled thioredoxins, thus providing a mechanism for autoregulation. Two cysteines essential for Yap1 oxidation are also essential for its activation by H₂O₂. The data are consistent with a model in which oxidation of Yap1 leads to disulfide bond formation with the resulting change of conformation masking recognition of the nuclear export signal by Crm1/Xpo1, thereby promoting nuclear accumulation of the protein. In sharp contrast to H₂O₂, diamide does not lead to the same Yap1 oxidized form and still activates mutants lacking cysteines essential for H₂O₂ activation, providing a molecular basis for differential activation of Yap1 by these oxidants. This is the first example of an H₂O₂-sensing mechanism in a eukaryote that exploits the oxidation of cysteines in order to respond rapidly to stress conditions.

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Introduction

Living cells have adaptive responses to oxidative stress, indicating that they sense increased levels of reactive oxygen species and respond to the signal by increasing the expression of defence activities. The mechanisms by which cells sense reactive oxygen species are relatively well understood in prokaryotes (Zheng and Storz, 2000), but not in eukaryotes.

The yeast transcription factor Yap1, a bZIP DNA-binding protein of the AP-1 family (Moye-Rowley *et al.*, 1989), is an essential regulator of the H₂O₂ adaptive response (Schnell *et al.*, 1992; Kuge and Jones, 1994) and controls many proteins of the H₂O₂ stimulon (Lee *et al.*, 1999a). These include most cellular antioxidants and enzymes of the glutathione and pentose phosphate pathways.

Yap1 is activated upon exposure to peroxides or to thiol-modifying drugs such as diamide (Kuge and Jones, 1994) by a mechanism acting at the level of subcellular protein

localization (Kuge *et al.*, 1997). Yap1 is restricted to the cytoplasm by virtue of rapid nuclear export by the export receptor Crm1/Xpo1 (Kuge *et al.*, 1998; Yan *et al.*, 1998). Upon exposure to oxidants, Yap1 accumulates in the nucleus due to loss of the Yap1–Crm1 interaction. Yap1 contains a short C-terminal domain (the CRD) carrying a non-canonical leucine rich nuclear export signal (NES) embedded in three repeated cysteine-containing motifs (see Figure 3A), which is important for the interaction with Crm1 (Kuge *et al.*, 1997, 1998; Yan *et al.*, 1998). Oxidation of these cysteines might modify the NES in a way that prevents recognition by Crm1, as suggested by (i) the constitutive cytoplasmic phenotype of a Yap1 allele bearing substitutions of all three C-terminal CRD cysteines (Kuge *et al.*, 1998), (ii) the dithiothreitol (DTT) enhancement of the co-precipitation of Crm1 with Yap1 (Yan *et al.*, 1998) and (iii) the sensitivity of the Yap1–Crm1 two-hybrid interaction to diamide (Yan *et al.*, 1998). Yap1 has another cluster of three cysteines (C303, C310, C315), but these appear not to be involved in Yap1–Crm1 interaction (Yan *et al.*, 1998). However, deletion mutagenesis has shown that a mutant protein lacking an internal segment between amino acids 225 and 335 was still inducible by diamide, but not by H₂O₂ (Wemmie *et al.*, 1997). This suggested the intriguing possibility that different mechanisms are used to activate Yap1 by these two oxidants, and that Yap1 N-terminal cysteines might be important for activation by H₂O₂ but not by diamide.

We sought to understand how H₂O₂ activates Yap1. We provide biochemical and genetic evidence that, in response to H₂O₂, Yap1 is activated by oxidation and deactivated by enzymatic reduction with Yap1-controlled thioredoxins, thus providing a mechanism for autoregulation. Two cysteines essential for Yap1 activation by H₂O₂ are likely to form a disulfide bond upon oxidation. In sharp contrast, diamide does not lead to the same oxidized form of Yap1 and still activates mutants lacking cysteines essential for the H₂O₂ response, thus providing a molecular basis for the differential activation of Yap1 by these oxidants. This is the first example of an H₂O₂-sensing mechanism in a eukaryote that exploits the oxidation of cysteines in order to respond rapidly to stress.

Results

Kinetics of the activation of Yap1 by H₂O₂

In response to 0.3 mM H₂O₂, the Yap1 target gene *TRX2* is expressed rapidly and transiently, with expression peaking at ~30 min and returning to baseline after 60 min (Figure 1A). The nuclear accumulation of a green fluorescent protein (GFP)–Yap1 fusion protein and its subsequent cytoplasmic redistribution paralleled the expression of *TRX2* (Figure 1B). Nuclear GFP–Yap1 staining was maximal 5 min after H₂O₂ treatment and was sustained for

Fig. 1. Kinetics of Yap1 activation. (A) Expression of *TRX2*. Total RNA was isolated from a wild-type strain, grown to an OD₆₀₀ of 0.3 in selective minimal medium, before and 10, 20, 30, 45 and 60 min after treatment with 300 μ M H₂O₂, and *TRX2* and *ACT1* (control gene) mRNAs were quantified by RT-PCR. An autoradiogram of the separated ³²P-labelled PCR products is shown in the upper panel and a plot of the *TRX2/ACT1* signal ratio quantified on a PhosphorImager in the lower panel. (B) Nuclear redistribution of Yap1. A Δ yap1 strain expressing GFP-Yap1 at an OD₆₀₀ of 0.3, treated with 300 μ M H₂O₂ for the time indicated and analysed for GFP staining. (C) Yap1 phosphorylation. Extracts of a Δ yap1 strain expressing Myc-tagged Yap1 (yMyc-Yap1), before and 5, 10, 15, 30, 45 and 60 min after treatment with 300 μ M H₂O₂ were immunoblotted with the anti-Myc monoclonal antibody after SDS-PAGE. (D) Yap1 phosphorylation correlates with Yap1 nuclear localization. Δ yap1 strain cultures expressing Myc-Yap1, Yap1^{L619S,L623S} or Yap1^{C598T,C620A,C629T}, before and 20 min after H₂O₂ treatment, were processed as in (C).

30 min. After 45 min, nuclear staining was half-maximal; after 60 min, it was indistinguishable from that of untreated cells. Yap1 activation also correlated with an altered migration of the protein on SDS-polyacrylamide gels. Within 5 min of H₂O₂ treatment, Myc-tagged Yap1 was shifted to more slowly migrating bands and this migration shift persisted for 1 h, being maximal at 30 min (Figure 1C). This migration shift must have been caused by protein phosphorylation, because it disappeared when extracts were treated with calf intestinal phosphatase (not shown). Yap1 phosphorylation could occur in the nucleus as suggested by (i) its kinetics, (ii) the constitutive phosphorylation of a constitutively nuclear mutant Yap1^{L619S,L623S} and (iii) its absence in the constitutively cytoplasmic mutant Yap1^{C598T,C620A,C629T} (Figure 1D). Therefore, although its role is not elucidated, phosphorylation of Yap1 correlates with its presence in the nucleus.

Yap1 becomes oxidized upon exposure to H₂O₂

The non-inducible nature of the cytoplasmic Yap1^{C598T,C620A,C629T} mutant demonstrated the importance of the C-terminal cysteines in Yap1 control and further suggested that Yap1 could be activated by direct oxidation. To test this hypothesis, we established a method to analyse directly the *in vivo* redox state of Yap1. If oxidation leads to covalent modification of Yap1, a change in conformation might be detectable by electrophoresis

under non-reducing conditions, as previously shown for OxyR (Tao, 1999). After H₂O₂ treatment, yeast cells were lysed with trichloroacetic acid (TCA) to protonate all free thiols and the precipitated proteins were re-solubilized with excess iodoacetamide to alkylate free thiols irreversibly and prevent their subsequent oxidation. As suspected, Yap1 from H₂O₂-treated cells migrated more quickly than the protein from untreated cells under non-reducing conditions (Figure 2A, lanes 1 and 2). However, this mobility shift was not seen when the extract was treated with 200 mM DTT prior to iodoacetamide (Figure 2A, lanes 3 and 4) or after electrophoresis under reducing conditions (Figure 2B). These data demonstrate that the more quickly migrating species observed under non-reducing conditions is an oxidized form of Yap1. Cellular treatment with *t*-butyl hydroperoxide, an organic peroxide, resulted in a similar oxidation-dependent electrophoretic migration shift (Figure 2A, lanes 5 and 6). We next performed a time-course analysis of the Yap1 redox status after H₂O₂ treatment. In this experiment, Yap1 was dephosphorylated *in vitro* to prevent an offset of the redox-dependent migration by the shift induced by phosphorylation. The oxidized form was observed as early as 2.5 min after H₂O₂ treatment and disappeared at 60 min (Figure 2B). The minimum concentration of H₂O₂ required to detect oxidized Yap1 was 100 μ M and the amount of the oxidized form increased with increasing

Fig. 2. *In vivo* oxidation of Yap1. (A) yMyc-Yap1 cultures (grown to an OD₆₀₀ of 0.4) before and after 2.5 min treatment with 400 μM H₂O₂ (lanes 1 and 2) or 1 mM *t*-butyl hydroperoxide (lanes 5 and 6) were lysed using TCA, treated with iodoacetamide and immunoblotted after non-reducing SDS-PAGE. Lanes 3 and 4 are the same as lanes 1 and 2 except that the TCA-precipitated protein pellet was dissolved in the presence of 200 mM DTT, before adding a 3.2 M excess of iodoacetamide. (B) Time course of Yap1 oxidation by H₂O₂. A yMyc-Yap1 culture before and 2.5, 5, 15, 30, 45 and 60 min after treatment with 400 μM H₂O₂, processed as in (A) (lanes 1 and 2), except that Yap1 was dephosphorylated and separated under non-reducing or reducing conditions as indicated. (C) The minimum H₂O₂ concentration required to oxidize Yap1 *in vivo*. A yMyc-Yap1 culture treated for 5 min with 25, 50, 100, 150, 200, 300 and 800 μM H₂O₂ and processed as in (A) (lanes 1 and 2). (D) Time-course analysis of Yap1 redox status in response to diamide. A yMyc-Yap1 culture before and 5, 15, 30, 45 and 60 min after treatment with 2 mM diamide, processed as in (A) (lanes 1 and 2) and separated under non-reducing or reducing conditions as indicated.

H₂O₂ concentration (Figure 2C). Therefore, the appearance of the oxidized form of Yap1 was well correlated with the kinetics of Yap1 activation.

Since diamide can also activate Yap1-target gene expression and Yap1 nuclear redistribution (Kuge and Jones, 1994; Kuge *et al.*, 1997), we monitored the *in vivo* redox status of Yap1 after treatment with this oxidant. Unexpectedly, ≤1 h after treatment with 2 mM diamide, a concentration able to activate Yap1 fully (not shown), we did not observe the redox-dependent migration shift of Yap1 observed on non-reducing PAGE after treatment with H₂O₂ (Figure 2D). The redox-dependent migration shift was not observed with higher concentrations of diamide (≤10 mM). These experiments indicate that treatment with H₂O₂ or *t*-butyl hydroperoxide, but not diamide, results in a distinctive oxidation-dependent change of conformation of Yap1 and suggest different modes of activation of Yap1 by these oxidants.

Cysteine residues important for the *in vivo* oxidation of Yap1

The residues important for the peroxide-induced redox-dependent mobility shift were identified by analysing Yap1 cysteine substitution mutants (Figure 3). The Yap1^{C598T,C620A,C629T} constitutive cytoplasmic allele and a mutant allele, Yap1^{C303A,C310A,C315A}, with alanine substitutions for all three N-terminal cysteine residues could not reproduce the redox-dependent mobility shift observed under non-reducing PAGE (Figure 3B), demonstrating that one or more cysteines of both the N- and C-terminal parts of the protein are required for the formation of the oxidized form of Yap1. Analysis of individual cysteine to alanine substitution mutants showed that of the six cysteines of Yap1, only C303 and C598 were required

for the occurrence of the oxidized form (Figure 3C). These two residues were also sufficient for the occurrence of the oxidized form of Yap1 since a mutant protein with substitution of all four other cysteines, Yap1^{C310T,C315A,C620A,C629T}, retained the redox-dependent mobility shift observed with the wild-type protein (Figure 3C). However, it is noteworthy that Yap1^{C310A} and Yap1^{C310T,C315A,C620A,C629T}, and, to a lesser degree, Yap1^{C629A}, could not be fully oxidized, suggesting that C310 might play an accessory role in the oxidation of C303 and C598.

Cysteine C303 and C598 are essential for the regulation of Yap1 by H₂O₂

The importance of C303 and C598 for the *in vivo* oxidation of Yap1 by H₂O₂ suggested that these two amino acid residues are essential for the regulation of Yap1 by this oxidant. This suggestion was tested by investigating the capacity of cysteine substitution mutants to rescue the H₂O₂ hypersensitivity of the *Δyap1* strain (Figure 4A). Yap1^{C315A} and Yap1^{C620A} had a wild-type H₂O₂ tolerance phenotype. All the other cysteine mutants were defective in this assay, with Yap1^{C303A} and Yap1^{C310A} displaying the highest sensitivity. The Yap1 cysteine mutants were also tested for their capacity to activate *TRX2* expression (Figure 4B and C) and to redistribute into the nucleus in response to H₂O₂ (Figure 5A). Again, in both assays, Yap1^{C315A} and Yap1^{C620A} had a wild-type phenotype. In contrast, Yap1^{C303A} and Yap1^{C598A} were unable to activate *TRX2* transcription and did not redistribute to the nucleus upon H₂O₂ treatment. Yap1^{C310A} and Yap1^{C629A} had a more complex phenotype. In these mutants, slight expression of *TRX2* was seen 1 h after H₂O₂ treatment. Yap1^{C310A} had an inefficient and delayed H₂O₂-induced redistribution

Fig. 3. Identification of Yap1 redox-active cysteines. **(A)** Schematics of Yap1. **(B)** Both N- and C-terminal cysteines are required to oxidize Yap1. *Yap1* strain cultures expressing Myc-Yap1, Yap1^{C303A,C310A,C315A} or Yap1^{C598T,C620A,C629T} before and 5 min after treatment with 400 μ M H₂O₂ were processed as in Figure 2A (lanes 1 and 2) and immunoblotted after non-reducing SDS-PAGE. **(C)** Cysteines C303 and C598 are the redox-active Yap1 cysteines. *Yap1* strain cultures expressing Myc-Yap1, Yap1^{C303A}, Yap1^{C310A}, Yap1^{C315A}, Yap1^{C598A}, Yap1^{C620A} or Yap1^{C629A}, and Yap1^{C310T,C315A,C620A,C629T} before and 5 min after H₂O₂ treatment, as in Figure 2B and immunoblotted after non-reducing or reducing SDS-PAGE, as indicated.

to the nucleus and Yap1^{C629A} had a partial constitutive nuclear phenotype but further concentrated in the nucleus after H₂O₂ treatment. The ability of cysteine mutants to redistribute to the nucleus was confirmed by their phosphorylation pattern (Figure 5B). Yap1^{C315A} and Yap1^{C620A} had a wild-type phosphorylation pattern whereas Yap1^{C303A} and Yap1^{C598A} did not undergo phosphorylation. Yap1^{C310A} and Yap1^{C629A} retained an inducible but lower phosphorylation level. Other Yap1 substitutions, C303S, C310S, C310T, C598S, C629S and C629T, were analysed and produced phenotypes similar to the corresponding C→A substitutions in all functional assays tested.

The critical role of C303 and C598 is at the level of the Crm1–Yap1 interaction

The data above demonstrate the critical role of the Yap1 N-terminal C303 and the C-terminal C598 in the activation of Yap1 by H₂O₂, probably due to their oxidation to a disulfide bond resulting in a change of conformation masking recognition of the Yap1 NES by Crm1. To provide further support for this model, we sought to evaluate whether the defective nuclear redistribution of Yap1^{C303A} and Yap1^{C598A} was due to their maintained

interaction with Crm1/Xpo1 under oxidative stress conditions, using a two-hybrid assay. As shown by Kuge *et al.* (1997) and Yan *et al.* (1998), Crm1 and full-length Yap1 interact in the two-hybrid assay. This interaction has also been shown to be sensitive to diamide (Yan *et al.*, 1998). We therefore evaluated the effect of H₂O₂ on the interaction between CRM1–LexA^{BD} and either full-length Yap1–B42^{AD}, Yap1^{C303A}–B42^{AD} or Yap1^{C598A}–B42^{AD} (Figure 6). Whereas the interaction of Crm1 with wild-type protein was, as expected, sensitive to H₂O₂, interaction of Crm1 with both Yap1^{C303A} and Yap1^{C598A} was not modified by this treatment. These data demonstrate that the effect of these two mutations is exerted at the level of the interaction between Yap1 and Crm1.

Yap1 is deactivated (reduced) by the thioredoxin system

The data presented demonstrate that activation of Yap1 by H₂O₂ involves the oxidation of the protein to a probable disulfide bond, indicating that this regulator might directly sense the increased levels of H₂O₂. In yeast, the H₂O₂-sensing mechanism might be coupled to the systems that control the cell redox status, as in *Escherichia coli* (Zheng *et al.*, 1998). To explore this possibility, Yap1 activation

Fig. 4. Activity of Yap1 cysteine substitution mutants. **(A)** Yap1 cysteine residues important for tolerance to H₂O₂. The *Δyap1* strains not expressing or expressing Myc-Yap1, Yap1^{C303A}, Yap1^{C310A}, Yap1^{C315A}, Yap1^{C598A}, Yap1^{C620A} or Yap1^{C629A} were tested for their tolerance to increasing concentrations of H₂O₂ by the patch assay on solid medium. **(B)** The same strains as in **(A)** were used to measure the amount of TRX2 and ACT1 mRNAs by RT-PCR as in Figure 1A. Cells were treated with 300 μM H₂O₂ for the time indicated. **(C)** The TRX2/ACT1 signal ratios quantified from the RT-PCR shown in **(B)** on a PhosphorImager were calculated and plotted as a function of time of exposure to H₂O₂.

was monitored in strains carrying mutations of the two main cellular thiol disulfide reducing pathways (Figure 7). The GSH pathway was tested with strains deleted for the glutathione reductase gene *GLR1* (*Δglr1*), or for both glutaredoxin genes *GRX1* and *GRX2* (*Δgrx1Δgrx2*), and with the *Δgsh1pro2-1Δglr1* strain. The latter strain lacks the γ-glutamyl cysteine synthase gene (*GSH1*), encoding the rate-limiting enzyme of glutathione synthesis, and the glutathione reductase gene (*GLR1*), and harbours a third mutation in the proline biosynthesis enzyme gene *PRO2*, which restores the synthesis of GSH to ~0.5% of the wild-type cellular content (our unpublished observations). The thioredoxin pathway was tested with strains lacking the thioredoxin reductase gene *TRR1* (*Δtrr1*), or both thioredoxin genes *TRX1* and *TRX2* (*Δtrx1Δtrx2*). The time-course-induced expression of *TRX2* and *TRR1* in response to H₂O₂ was similar to that of the wild-type strain in *Δglr1*, *Δgsh1pro2-1Δglr1* (Figure 7A and B) and *Δgrx1Δgrx2* (not shown). By contrast, in strains inactivated in the thioredoxin pathway, Yap1 was constitutively partially active (~20–50%), further activated after H₂O₂

treatment and remained maximally active after 2 h. This deregulated gene expression was specific to Yap1, since only the proteins of the Yap1 regulon were constitutively high in *Δtrx1Δtrx2*, as shown by two-dimensional gel electrophoresis (data not shown). We also monitored Yap1 activity in a strain deleted for *TSA1* (*Δtsa1*), which encodes a thioredoxin-dependent thiol peroxidase (Chae *et al.*, 1994). In this strain, Yap1 was not activated constitutively, but activation by H₂O₂ was prolonged (Figure 7A and B). However, Yap1 activity still decreased to baseline levels after ~1.5 h, suggesting that the deregulation of Yap1 observed in the thioredoxin mutants is not solely related to a strain-intrinsic peroxide-scavenging defect, but to a lack of a direct effect of thioredoxins on the protein redox status. Consistent with the deregulated expression of Yap1-target genes, Yap1 was constitutively partially oxidized in *Δtrr1* and *Δtrx1Δtrx2* (Figure 7C). Deregulation was also correlated with a constitutive nuclear localization of Yap1 in *Δtrr1* and *Δtrx1Δtrx2*, but not in mutants of the GSH pathway or in the *Δtsa1* strain (Izawa *et al.*, 1999; data not shown).

Fig. 5. Essential role of C303 and C598 for H₂O₂-induced Yap1 nuclear redistribution. **(A)** Analysis of the cellular distribution of GFP-tagged Yap1^{C303A}, Yap1^{C310A}, Yap1^{C598A} and Yap1^{C629A}. Cells taken before or 12 min after treatment with 300 µM H₂O₂ were analysed for GFP or DAPI staining or under visible light. **(B)** Phosphorylation of Yap1 cysteine mutants as an indicator of their subcellular localization. $\Delta yap1$ cultures expressing Myc-Yap1, Yap1^{C303A}, Yap1^{C310A}, Yap1^{C315A}, Yap1^{C598A}, Yap1^{C620A} or Yap1^{C629A} were taken before and after treatment with 300 µM H₂O₂ and processed as in Figure 1C.

Because thioredoxins are known to catalyse protein disulfide bond reduction by NADPH-dependent thioredoxin reductase, they could catalyse the reduction (deactivation) of oxidized Yap1. To test this hypothesis, we incubated extracts containing oxidized Yap1 with increasing amounts of thioredoxin, thioredoxin reductase and NADPH. The oxidation-dependent shift of migration was completely eliminated within 30 min in the presence of 20 µM thioredoxin (Figure 7D).

Discussion

Yap1 as the yeast H₂O₂ sensor

The data presented here establish that the Yap1 transcription factor is activated by oxidation within minutes of H₂O₂ treatment and therefore fulfils the function of a redox sensor. These data also provide evidence for an

oxidation-induced change of conformation of Yap1, and the requirement of residues C303 and C598 in its formation. This requirement, and the lack of H₂O₂ reactivity of Yap1^{C303A} and Yap1^{C598A}, establish that C303 and C598 are part of the Yap1 redox centre. Although a glutathione mixed disulfide or a sulfenic acid are possible cysteine oxidation products, C303 and C598 probably oxidize to form an intramolecular disulfide bond. The prolonged activation of Yap1 in mutants of the thioredoxin pathway and the ability of thioredoxin to catalyse Yap1 reduction *in vitro* indicate that the thioredoxin system acts to deactivate (reduce) Yap1, which is consistent with the presence of a disulfide bond in its oxidized form. Since both *TRX2* and *TRR1* are targets of Yap1, activation of the pathway by H₂O₂ is auto-regulated. These data substantiate the presence of a disulfide bond in oxidized Yap1, but the participation of

Fig. 6. The critical role of C303 and C598 is at the level of Crm1–Yap1 interaction. β -galactosidase production from a *lacZ* reporter gene was assayed in strain EGY48 carrying pKW442 (*Crm1-lexA^{BD}*) or pEG202 (empty vector) and one of the following: pLDB439 (YAP1-B42^{AD} or pYAP1^{C303A}-B42^{AD}), pYAP1^{C598A}-B42^{AD} or pJG4-5 (empty vector). (A) Assay performed on solid medium in the absence or presence of H₂O₂ at the concentration indicated. (B) Assay performed on liquid medium in the absence (–) or presence (+) of 500 μ M H₂O₂. The results are the averages of four independent experiments.

a metal or another prosthetic group in its redox-active centre cannot be ruled out. Of the other cysteines of Yap1, C315, C620 and C629 do not appear essential for redox sensing. However, C620 and C629 might be important in Yap1 control by being close to or part of the NES (Kuge *et al.*, 1998; Yan *et al.*, 1998; Coleman *et al.*, 1999). In the case of C620, an alanine substitution appears to be tolerated for NES function (this work), whereas a threonine substitution is not (Kuge *et al.*, 1997). Similarly, an alanine substitution of C629 partially disrupts the NES function (Coleman *et al.*, 1999; this work) while a threonine substitution has a wild-type phenotype (Kuge *et al.*, 1997). The depressed activity of Yap1^{C29A} is similar to the previously observed, but not understood, decreased activity towards H₂O₂ of all nuclear constitutive Yap1 alleles tested (Kuge *et al.*, 1997; Coleman *et al.*, 1999; data not shown). The role of C310 is more complex. As the corresponding substitution mutant can reproduce the oxidation-dependent electrophoretic migration shift and is still capable of redistributing to the nucleus upon H₂O₂ treatment, this cysteine residue is dispensable for redox sensing. One remarkable phenotype of Yap1^{C310A} is its inefficient oxidation and delayed nuclear redistribution, which suggests that C310 could have a role in the formation of the oxidized conformation, perhaps by modifying the vicinal electronic environment if this residue becomes partially oxidized to a sulfenate upon exposure to H₂O₂. However, the profoundly decreased activity of Yap1^{C310A} may also indicate another undefined functional role for the domain containing this residue (Coleman *et al.*, 1999). Our data also showed that Yap1 becomes phosphorylated upon exposure to H₂O₂. This modification occurs outside the C-terminal CRD since it was still observed in a Yap1 truncated version lacking this domain (not shown). As it did not occur in mutants unable to redistribute to the nucleus, and was constitutive in a constitutive nuclear mutant (Figure 1), this modification probably occurs in the nucleus, and therefore at a step after protein oxidation, which is the critical event triggering Yap1 nuclear redistribution. Although its role is not understood, phosphorylation could be important by

providing a regulated interaction with other substrates or as a marker for protein degradation.

The data presented here establish that activation of Yap1 by H₂O₂ involves the critical oxidation of the N-terminal CRD C303 and the C-terminal CRD C598 probably to a disulfide bond, with the resulting change of conformation masking recognition of the Yap1 NES by Crm1/Xpo1, thereby promoting accumulation of Yap1 into the nucleus. This model would provide a new example of a protein regulated by reversible disulfide bond formation, as a way to respond rapidly to oxidative stress (Åslund and Beckwith, 1999).

The mechanisms by which H₂O₂ and diamide activate Yap1 are different

The model proposed above seems to contradict studies that analysed the activation of Yap1 by diamide (Kuge *et al.*, 1997, 1998; Yan *et al.*, 1998). In particular, Kuge *et al.* (1997) showed that the Yap1 C-terminal CRD by itself could confer regulated nuclear redistribution to a reporter protein in response to diamide, while Yan *et al.* (1998) showed that any one of the C-terminal cysteines was required for the diamide regulation of Yap1. These published data clearly rule out involvement of disulfide bond formation between N- and C-terminal cysteine in the activation of Yap1 by diamide. This apparent contradiction could indicate, as pointed out by Wemmie *et al.* (1997), that there is a difference in the mechanism by which H₂O₂ and diamide activate Yap1. This is now clearly demonstrated by lack of the H₂O₂-induced redox-dependent migration shift upon diamide treatment (Figure 2), indicating that this oxidant does not generate the H₂O₂ oxidation product of C303 and C598. In addition, Yap1^{C303} and Yap1^{C598} still redistributed to the nucleus in response to diamide (data not shown), which demonstrates the specificity of their defective H₂O₂ response, and is consistent with the notion that any one cysteine can fulfil the required regulatory function needed in this process (Yan *et al.*, 1998). In conclusion, we propose that diamide may activate Yap1 by direct modification of one or more C-terminal cysteines, perhaps by creation of a stable

Fig. 7. Deactivation (reduction) of Yap1 by the thioredoxin system. (A and B) Expression of *TRX2* (A) and *TRR1* (B) in wild-type, $\Delta trr1$ (A), $\Delta trx1\Delta trx2$ (B), $\Delta gsh1\Delta glr1pro2-1$ and $\Delta tsa1$ strains. Cells were treated with 300 μM H_2O_2 for the indicated time and processed for RT-PCR as in Figure 1A. The *ACT1* autoradiograms are not shown. (C) Yap1 is oxidized in mutants of the thioredoxin system. yMyc-Yap1, $\Delta trx1\Delta trx2$ and $\Delta trr1$ expressing Myc-Yap1 (grown to an OD_{600} of 0.3) were processed as in Figure 2A and extracts were analysed by immunoblotting after non-reducing PAGE. (D) Reduction of oxidized Yap1 by the thioredoxin system. yMyc-Yap1 (grown to an OD_{600} of 0.4), treated with 400 μM H_2O_2 , lysed with glass beads in 100 mM Tris-HCl pH 8, 0.1% SDS, 1 mM EDTA, complete protease inhibitors and PMSF. Twenty-five micrograms of extracts were incubated with 1, 10 or 20 μM thioredoxin (from *Spirulina* sp.), 1.3 μM *E.coli* thioredoxin reductase and 1 mM NADPH (Sigma) for 30 min at 37°C. Iodoacetamide (75 mM) was added and the samples were analysed by immunoblotting after non-reducing PAGE.

sulphenylhydrazine thereby modifying the vicinal NES and, hence, the recognition of Yap1 by Crm1.

Conservation of the H_2O_2 -sensing mechanism between eukaryotes and prokaryotes

The two best characterized oxidative stress sensors are the *E.coli* transcriptional regulators OxyR and SoxR. SoxR is activated by reversible one-electron oxidation of its iron-sulfur centres in response to superoxide-generating compounds or nitric oxide (Gaudu *et al.*, 1997; Hidalgo *et al.*, 1997). OxyR is directly activated by H_2O_2 through formation of an intramolecular disulfide bond, and is deactivated by glutaredoxins (Zheng *et al.*, 1998). Therefore, Yap1 and OxyR appear functionally homologous. Both activators control activities important for cellular thiol redox control and for scavenging reactive oxygen species (Lee *et al.*, 1999a; Zheng and Storz, 2000) and share the function of sensing increased H_2O_2 levels. The quite different molecular control of these regulators,

which is exerted at the level of the subcellular localization of the protein in the case of Yap1 (Kuge and Jones, 1997) and at the level of promoter selection and DNA binding in the case of OxyR (Toledano *et al.*, 1994), reflects the greater cellular complexity in eukaryotes compared with prokaryotes. However, the biochemical modifications that reversibly activate these regulators, involving disulfide bond formation and deactivation by a thioltransferase system, appear to be conserved. Furthermore, although OxyR is preferentially deactivated by the glutathione pathway and Yap1 by the thioredoxin pathway, in both cases the whole system is autoregulated since each factor is deactivated by its target genes. It will still be important to demonstrate directly the presence of a disulfide bond in oxidized Yap1, and whether Yap1, like OxyR (Åslund *et al.*, 1999), is directly oxidized by H_2O_2 . We indeed do not rule out the possibility that another molecule acts to transduce the H_2O_2 redox potential to Yap1, in the same way as protein disulfide isomerase transmits the reducing

equivalent generated during photosynthesis to the chloroplast polyadenylate binding protein, cPABP (Kim and Mayfield, 1997).

Yap1 does not sense GSH/GSSG status

Apart from their conserved features, significant differences could exist between the prokaryotic and eukaryotic redox sensing systems. OxyR can sense the cellular thiol–disulfide status, in addition to sensing H₂O₂. This disulfide stress sensing is based on OxyR constitutive activation in *E. coli* mutants simultaneously compromised in both thioredoxin and GSH pathways, and was proposed to explain OxyR activation by diamide (Åslund *et al.*, 1999). However, the data presented here do not support that conclusion for Yap1. We observed that Yap1 is only constitutive in mutants of the thioredoxin pathway, but not in mutants of the GSH pathway. This could be interpreted as meaning that, in yeast, the drop in the cellular redox status needed to activate Yap1 is achieved after inactivation of the thioredoxin but not the glutathione pathway. However, this possibility can be ruled out by the status of the thiol–disulfide balance, which is more oxidized in mutants of the GSH pathway. Indeed, the percentage of oxidized GSH (GSSG), which is thought to be the best index of the cellular thiol redox balance, was 54% in $\Delta glr1$ compared with 22% in $\Delta trx1\Delta trx2$; in wild type the proportion of GSSG was <5% (not shown), as shown previously (Muller, 1996). Alternatively, the constitutive activation of Yap1 in mutants of the thioredoxin pathway could be interpreted as the sole consequence of the defective enzymatic deactivation of oxidized protein, which accumulates as a result of endogenous production of reactive oxygen species, and not as re-equilibration of Yap1 with an oxidizing cellular redox environment. This hypothesis would be consistent with the idea that diamide, although dramatically shifting the GSH/GSSG ratio towards its oxidized form (Kosower and Kosower, 1995; data not shown), activates Yap1 not through this perturbation, as proposed for OxyR (Åslund *et al.*, 1999), but directly, as discussed above. We conclude that Yap1 has evolved to sense increased peroxide levels but not the GSH redox balance.

Materials and methods

Strains and culture conditions

Most of the experiments were performed with the wild-type strain YPH98 (Sikorski and Hieter, 1989) (*Mat α* *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1 Δ leu2 Δ 1*) and its isogenic derivatives. The $\Delta yap1$, $\Delta glr1$, $\Delta tsal$, $\Delta gsh1$ and $\Delta trr1$ strains were described previously (Lee *et al.*, 1999b). $\Delta trx1\Delta trx2$ was prepared by a one-step amplification protocol that successively replaced the entire *TRX1* and *TRX2* open reading frames (ORFs) with the *TRP1* and kanamycin genes, respectively. $\Delta gsh1pro2-1\Delta glr1$ is the $\Delta gsh1$ strain carrying a *PRO2* mutation that rescues the strain's GSH auxotrophy by allowing the synthesis of traces of GSH (0.5% of the wild-type GSH content as determined by a microbiological assay and by thin layer chromatography after [³⁵S]methionine pulse labelling) and a deletion of the entire *GLR1* ORF replaced by *URA3*. The *pro2-1* suppressor mutation acts by diverting the γ -glutamate synthesized in the first step of the proline biosynthetic pathway into the synthesis of GSH (D.Spector and M.B.Toledano, unpublished observations). Given the GSH auxotrophy of the $\Delta gsh1$ mutation, the *pro2-1* suppressor mutation ensures the presence of very low and constant levels of GSH on minimal medium. $\Delta grx1$, $\Delta grx2$ and the double deletion $\Delta grx1\Delta grx2$ are not isogenic with YPH98 and were a gift from Dr C.Grant (UMIST, Manchester) (Luikenhuis *et al.*, 1998). EGY48 (*Mat α* *ura3-52 his3*

LEXA_{opp(X6)}-LEU2 <pSH18-34 *URA3* 2 μ >) (R.Brent) was used for the two-hybrid assay. The minimal medium (SD) was yeast nitrogen base (6.7 g/l) and dextrose (20 g/l) supplemented with the required amino acids. Dextrose can be replaced by 2% raffinose (S raffinose) or 2% galactose (S galactose). The H₂O₂ sensitivity assays were performed as described (Lee *et al.*, 1999a).

Preparation of cell extracts and western blot analysis

Pelleted cells were washed in 20% TCA, frozen and lysed by vortexing after adding glass beads and 12.5% TCA. The lysate was centrifuged and the precipitated pellet dissolved in 2% SDS, 5% β -mercaptoethanol, 62.5 mM Tris–HCl pH 8.7, 10% glycerol, 0.01% bromophenol blue, boiled and loaded on a 10% SDS–polyacrylamide gel (30:0.4 acrylamide:bisacrylamide). To monitor the Yap1 redox state, the TCA-precipitated pellet was washed in acetone, air dried, dissolved in 75 mM iodoacetamide, 1% SDS, 100 mM Tris–HCl pH 8, 1 mM EDTA, complete protease inhibitors (Boehringer Mannheim) and incubated at 25°C for 15 min. Where indicated, Yap1 was dephosphorylated with 20 U calf intestinal phosphatase (New England Biolabs) for 45 min at 37°C, after dialysis of the resuspended iodoacetamide-treated protein pellet for 4 h against 10 mM Tris–HCl pH 8, 50 mM NaCl, 10 mM MgCl₂. Protein extracts were separated by non-reducing SDS–PAGE after adding 3 $\times$ Laemmli sample buffer not containing β -mercaptoethanol and boiling. The separated proteins were blotted on to a nitrocellulose membrane and probed with the 9E10 anti-Myc monoclonal antibody; bound antibodies were detected using an enhanced chemiluminescence western blotting reagent kit (Amersham Pharmacia).

Analysis of cell GFP staining by immunofluorescence microscopy

Five minutes after addition of DAPI, cells were centrifuged, washed, resuspended in DABCO (75% glycerol, 0.25 $\times$ phosphate-buffered saline, 200 mM diazabicyclooctane) and analysed with a Leica DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled CCD camera and MetaMorph software (Universal Imaging, Inc.).

Two-hybrid assay

EGY48 carrying pSH18-34 (2 μ plasmid carrying a *lacZ* reporter gene under the control of eight *lexA* operators) (Gyuris *et al.*, 1993) was transformed with PKW442 [CRM1–LexA^{BD} (Stade *et al.*, 1997)] or pEG202 (*lexA*_(1–202)) and one of the following: pJG4-5 [B42^{AD} haemagglutinin (HA) epitope tag TRP1], pLDB439 [YAP1–B42^{AD}: full-length Yap1 cloned between the *EcoRI* and *XhoI* sites (Yan *et al.*, 1998) of pJG4-5], pYAP1^{C303A}–B42^{AD} or pYAP1^{C598A}–B42^{AD}. The C303 and C598 mutations were introduced by cloning into YAP1–B42^{AD} a mutagenized fragment generated by the two-step PCR method and inserted in pLDB439 between the *BamHI* and *PacI* sites (C303) or between the *PacI* and *XhoI* sites (C598). To test interaction in liquid medium, cells pre-grown in 2% S raffinose lacking uracil, histidine and tryptophan were re-inoculated in the same medium to an OD₆₀₀ of 0.3–0.5 and were transferred to an equivalent volume of 2% S galactose for 30 min. Cells were then treated or not with H₂O₂ (500 μ M) for 1 h. The β -galactosidase assay was performed as described (Miller *et al.*, 1972). To test interaction on solid medium, cells grown in 2% S raffinose were patched at a density of 4.5 $\times$ 10⁷ cells on solid 1% S raffinose/2% galactose medium and incubated for 2.5 h at 30°C. The assay was revealed by overlaying the cells with top agar containing X-gal (0.04%).

Construction of plasmids

Myc-Yap1 contains a Myc₉ epitope tag inserted at the unique *AccI* site, three codons downstream from the ATG, and is expressed from the centromeric pRS316 plasmid (Lee *et al.*, 1999b). A 1.17 kb fragment encoding the C303A, C310A and C315A substitutions, amplified from Pldb583 (a gift from L.Davis, Brandeis University) (Yan *et al.*, 1998), was recombined to Myc-Yap1 by gap repair, resulting in Yap1^{C303A,C310A,C315A}. A 327 bp *NdeI*–*PacI* fragment of Pldb518 encoding the C598T, C620A and C629T substitutions (Yan *et al.*, 1998) was subcloned into Myc-Yap1, resulting in Yap1^{C598T,C620A,C629T}. Yap1^{L619S,L623S}, Yap1^{C303A}, Yap1^{C310A}, Yap1^{C598A}, Yap1^{C620A}, Yap1^{C629A} and Yap1^{C315A} were prepared by a two-step PCR method and the mutagenized fragments were introduced into Myc-Yap1 by gap repair. GFP-Yap1 is a GFP N-terminal fusion expressed from vector pRS cp-GFP HA-YAP1 GFP (*TRP1*, *CEN6*–ARSH4) (a gift from S.Kuge, University of Tokyo) (Kuge *et al.*, 1997). GFP-Yap1 fusions carrying individual C $\rightarrow$ A mutations were prepared by subcloning the corresponding mutagenized fragments into the *BamHI* and *BstEII* sites of pRS

cp-GFP HA-YAP1. All the constructs used in this study were sequenced completely.

Quantitative RT-PCR assays

Total RNA was isolated as described by Lee *et al.* (1999b). RT-PCR assays were performed as described by Godon *et al.* (1998). cDNAs were synthesized by random hexanucleotide-primed reverse transcription from 1 µg of total RNA. Quantitative PCR was performed using primers specific for *TRX2* (CGTCACTCAATTAATAATCCGCTTC, GGAAGC-AATAGCTTGCTTGCTTGATAGC), *TRR1* (CCAGGGCAGAAA-TCAAGCCAATCC, ATAAACCGCTGACTGGCAAATCGG) and *ACT1* (TTGGATTCCGGTGATGGTGTACT, TGAAGAAGATTG-AGCAGCGGTTTG). ³²P-labelled PCR products were separated on a native polyacrylamide gel, dried, exposed to autoradiography film and quantified on a PhosphorImager.

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