Deletion of the *Saccharomyces cerevisiae* TRR1 Gene Encoding Thioredoxin Reductase Inhibits p53-dependent Reporter Gene Expression*

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George D. Pearson and Gary F. Merrill
From the Department of Biochemistry and Biophysics and the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, Oregon 97331

The prevalence of *p53* gene mutations in many human tumors implies that *p53* protein plays an important role in preventing cancers. Central among the activities ascribed to *p53* is its ability to stimulate transcription of other genes that inhibit cells from entering *S* phase with damaged DNA. Human *p53* can be studied in yeast where genetic tools can be used to identify proteins that affect its ability to stimulate transcription. Although *p53* strongly stimulates reporter gene expression in wild type yeast, it only weakly stimulates reporter gene expression in *Δtrr1* yeast that lacked the gene encoding thioredoxin reductase. Furthermore, ectopic expression of *TRR1* in *Δtrr1* yeast restored *p53*-dependent reporter gene activity to high levels. Immunoblot assays established that the *Δtrr1* mutation affected the activity and not the level of *p53* protein. The results suggest that *p53* can form disulfides and that these disulfides must be reduced in order for the protein to function as a transcription factor.

Mutations in the *p53* tumor suppressor gene have been found in more than half of human cancers. Of 5000 mutations documented, about 85% are missense mutations and greater than 95% are found in the DNA-binding core domain. Ectopic expression of wild type *p53* in cells with *p53* null mutations suppresses cell growth; expression of oncogenic forms does not (1). Moreover, oncogenic forms of *p53* inhibit the ability of wild type *p53* to stimulate reporter genes (2). Human tumors frequently express large amounts of *p53* antigen. Mice homozygous for *p53* null mutations develop normally but show a high tumor incidence (3). These and other results suggest that although *p53* null mutations are oncogenic, oncogenicity is most frequently due to dominant negative mutations that result in production of a defective *p53* that interferes with the function of wild type *p53*.

It is becoming increasingly clear that cancer results from defects in cell cycle control. Our understanding of cell cycle control derives largely from work first initiated in yeast. All eucaryotic cells have cyclins and cyclin-dependent protein kinases (CDKs) that initiate and orchestrate the transition from one cell cycle compartment to the next. In addition to CDKs, all cells have checkpoint systems that prevent CDKs from initiating the next cell cycle phase until critical processes in the current phase are complete (4). One major checkpoint mechanism is mediated by a set of proteins called cyclin-dependent kinase inhibitors, which bind and inactivate CDKs.

In both yeast and mammalian systems, initiation of *S* phase is delayed when *G1* cells are UV-irradiated. In mammalian cells lacking *p53* (or in cells with dominant negative *p53*), the delay in *S* phase does not occur, and cells enter *S* phase with damaged DNA. If wild type *p53* is present, the cells enter *S* phase only when the damage is repaired, or, if the damage is too severe, the cells undergo apoptosis. The discovery that *p53* stimulates transcription of the cyclin-dependent kinase inhibitor gene *CIP1*, which encodes a G1 cyclin/CDK inhibitor, in response to DNA damage provides a molecular understanding of the basis for *S* phase delay. The *CIP1* upstream region contains a *p53*-binding site, and accumulation of Cip1 activity and cell cycle arrest in irradiated cells requires wild type *p53* (5).

Although yeast does not contain a *p53* gene, the similarity of cell cycle control mechanisms and transcriptional mechanisms in all eucaryotic organisms prompted investigators to ask whether human *p53* was active in yeast. *p53* binds the consensus RRRC(A/T)(A/G)YYY (6). Reporter genes equipped with multiple copies of the consensus site are stimulated by ectopic expression of *p53* not only in mammalian cells (2, 6) but in yeast (7, 8) as well. *p53* activity is also affected by yeast mutations. Koerte et al. (9) screened for yeast mutations that created a requirement for human *p53*. They identified a gene, termed *RFP1* (requires fifty-three protein), that results in cell cycle arrest at G2/M when inactivated. Thiagalingam et al. (8) screened for yeast mutations that prevented *p53* transactivation of a *p53*-responsive *LacZ* reporter gene. Using one of the obtained mutants, they identified a high copy suppressor, termed *PAK1* (*p53* activating kinase), that encoded a protein with protein kinase motifs. They did not establish that Pak1 actually phosphorylated *p53*. Nigro et al. (10) investigated whether overexpression of *p53* blocked cell cycle progression in *Saccharomyces cerevisiae*. Using a high copy plasmid and a galactose-inducible promoter they did not observe significant inhibition of growth by *p53*. However, by using a protease-deficient strain and by cotransforming with a plasmid encoding the human CDK p34cdc2, they showed that human *p53* strongly inhibited yeast growth. Although human p34cdc2 was required to see strong growth inhibition by *p53*, it did not affect the phosphorylation state of *p53* protein, suggesting that p34cdc2 acted indirectly, presumably by phosphorylating other yeast proteins.

The above studies were done with the budding yeast *S. cerevisiae*. A recent study, done with the fission yeast *Schizosaccharomyces pombe*, prompted our investigation of the involvement of thioredoxin reductase in *p53* activity. Casso and Beach (11) found that high level expression of *p53* in *S. pombe* arrested cell growth. A mutant circumventing growth arrest was identified, and the wild type gene, a homolog of thioredoxin

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reductase, was cloned by complementation. To determine whether the S. pombe results reflected a conserved mechanism whereby the ability of p53 to stimulate transcription requires thioredoxin reductase activity, the effect of deleting the thioredoxin reductase gene on p53-dependent reporter gene expression was investigated in the evolutionarily distant organism S. cerevisiae. The results showed that the transactivating activity of p53 in budding yeast also required thioredoxin reductase. The data suggest that p53 may be subject to redox regulation.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast strain MY301 (mat-a ade2 ura3 leu2 trp1 his3 Δtrr1: HIS3 bar1) was derived by crossing MY199 (12) to W303–1 (13) and serially backcrossing Δtrr1:HIS3 segregants to W303–1 or ST2626 (a bar1 derivative of W303–1). Inheritance of the Δtrr1:HIS3 allele was confirmed by Southern blot analysis and hydrogen peroxide sensitivity. MY301 was temperature-sensitive for growth and auxotrophic for methionine. Yeast carrying effector and reporter plasmids were derived and grown at 25 °C in minimal medium consisting of yeast nitrogen broth, 2% glucose, and required supplements.

Plasmids—pRS314, pRS315, and pRS316 are ARS/CEN vectors marked respectively with TRP1, LEE2, and URA3 (14). The plasmid expressing thioredoxin reductase (pRS316-TRR1) was derived by inserting a 2.3-kilobase pair EcoRI fragment containing the TRR1 coding region and flanking regions into pRS316. The plasmid expressing human p53 (DO-1) mouse antibody at a dilution of 1:10,000. X-ray films of the blots were scanned and quantitated using ImageQuant software (Molecular Dynamics).

RESULTS

Thioredoxin Reductase Is Required for p53-dependent Reporter Gene Expression in Budding Yeast—Prompted by the work implicating thioredoxin reductase in the growth-inhibitory and transcriptional action of p53 in S. pombe (11), we investigated the effect of thioredoxin reductase gene mutations on p53 transcriptional activity in S. cerevisiae. A plasmid encoding a p53-responsive LacZ reporter gene (pRS315-β-gal) and an effector plasmid encoding human p53 under the control of the constitutive PGK promoter (pRS314-p53) were introduced into strains that carried either a wild type (TRR1) or a null (Δtrr1) allele of the gene encoding thioredoxin reductase. Table I summarizes the results of the β-galactosidase assays. Reporter gene expression was high in transformants of the TRR1 strain. In contrast, Δtrr1 transformants had more than 10-fold lower but nevertheless detectable levels of β-galactosidase activity. Furthermore, reporter gene activity required p53 expression because β-galactosidase activity could not be detected in transformants of either the wild type strain or the null mutant strain when the p53-effector plasmid was replaced with the empty pRS314 vector.

We next investigated whether the TRR1 gene carried on a single copy plasmid could rescue strong reporter gene expression in the Δtrr1 strain. Either a plasmid expressing thioredoxin reductase (pRS316-TRR1) or the empty vector (pRS316) was introduced together with p53-expressing effecter (pRS314-p53) and p53-responsive LacZ reporter (pRS315-β-gal) plasmids into the null mutant strain. The results of the β-galactosidase assays are summarized in Table II. Ectopic expression of thioredoxin reductase from the single copy TRR1 gene carried on the plasmid restored high levels of β-galactosidase activity in transformants of the Δtrr1 strain. When the plasmid expressing thioredoxin reductase was replaced with the empty pRS316 vector, more than 10-fold lower reporter gene expression was again found in transformants of the null mutant strain. Therefore, p53-dependent reporter gene expression required a functional S. cerevisiae thioredoxin reductase gene.

Characterization of p53 Requirement for Thioredoxin Reductase—The observation that p53 cannot transactivate a p53-responsive reporter gene in yeast lacking thioredoxin reductase could have two basic explanations. In the absence of thioredoxin reductase, either the steady state levels of p53 protein could be reduced or the activity of p53 itself could be inhibited. To investigate the effect of the Δtrr1 mutation on the amount of p53 protein in S. cerevisiae, p53 levels were determined by immunoblot analysis as shown in Fig. 1A. The wild type TRR1 strain transformed with (lanes 1–3) or without (lane 4) the p53-expressing effector plasmid served respectively as positive and negative controls for the Western blot assay. Quantitative densitometry established that two Δtrr1 transformants lacking thioredoxin reductase (lanes 6 and 8) had just as high or higher p53 protein levels as two other Δtrr1 transformants ectopically expressing thioredoxin reductase (lanes 5 and 7). Because p53 protein levels clearly remained unaffected in the absence of thioredoxin reductase, it is not possible that the lack of thioredoxin reductase activity in the Δtrr1 strain might interfere with assays for the enzymatic activity of β-galactosidase, the levels of β-galactosidase protein were also determined by immunoblot analysis as shown in Fig. 1B. Quantitative densitometry established that the two Δtrr1 transformants ectopically expressing thioredoxin reductase (lanes 1 and 3) had more than 10-fold higher β-galactosidase protein levels than the two other Δtrr1 transformants lacking thioredoxin reductase (lanes 2 and 4), in good agreement with the enzymatic assays (Table II).

DISCUSSION

The results presented here show that transactivation of a β-galactosidase reporter gene by human p53 was markedly inhibited in a S. cerevisiae Δtrr1 mutant lacking thioredoxin reductase. Ectopic expression of thioredoxin reductase from a single copy plasmid in the Δtrr1 mutant circumvented the
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Table II

| Plasmid*        | β-Galactosidase activity |
|-----------------|--------------------------|
| pRS316-TRR1     | 205 ± 80 (4)*            |
| pRS316          | 15 ± 8 (3)               |

* In addition to the indicated plasmid, all transformants contained the p53-expressing effector plasmid pRS314-p53 and the p53-responsive LacZ reporter plasmid pRS315-β-gal.

β Number of transformants assayed.

Fig. 1. Immunoblot analyses of p53 and β-galactosidase proteins in W303 (TRR1) and MY301 (Δtrr1) strains. The presence or absence of p53 or thioredoxin reductase in transformants is indicated by + or −, respectively. Equal amounts of total yeast proteins from the indicated transformants were resolved by gel electrophoresis and transferred to nitrocellulose. A, immunoblot probed with an antibody against p53. Lanes 1–3, increasing amounts (0.1-, 0.3-, and 1.0-fold, respectively) of a wild type TRR1 transformant carrying pRS314-p53 and pRS315-β-gal. Lane 4, a wild type TRR1 transformant carrying pRS314 and pRS315-β-gal. Lanes 5 and 7, two different Δtrr1 mutant transformants carrying pRS314-p53, pRS315-β-gal, and pRS316-TRR1. Lanes 6 and 8, two different Δtrr1 mutant transformants carrying pRS314-p53, pRS315-β-gal, and pRS316. B, immunoblot probed with an antibody against β-galactosidase (β-Gal). Lanes 1–4 correspond to lanes 5–8 in A above.

Fig. 2. Structure of the core domain of the p53 protein showing the surface methionine (Met846) and five cysteines (Cys176, Cys182, Cys229, Cys242, and Cys277) in yellow. DNA binds to the lower surface with the helix axis roughly perpendicular to the page.

Inhibition and restored strong p53-dependent transactivation. Inhibition of reporter gene expression was not due to diminished amounts of p53 protein. Because thioredoxin reductase was also found necessary for p53 activity in S. pombe (11), an organism estimated to be separated evolutionarily from S. cerevisiae by one billion years, the thioredoxin reductase requirement likely reflects a conserved mechanism also operating in humans. Thioredoxin reductase is a protein disulfide reductase. Our results thus suggest that p53 can form disulfides and that these disulfides must be reduced in order for the protein to function as a transcription factor. Recent biochemical experiments have shown that the pharmacological application of oxidizing and reducing agents can modulate the biological activities of murine p53 (17) and human (18) p53. In particular, treatment of p53 with reducing agents enhances sequence-specific DNA binding (17).

Human p53 is a tetrameric phosphoprotein. The 393-residue polypeptide contains five conserved regions (I–V) that are nearly identical in all vertebrates analyzed. Biochemical and reverse genetic experiments have defined three functional domains. The proline-rich, acidic N-terminal domain (residues 1–43) contains conserved region I, has been shown to interact with TATA-binding protein, and is involved in transcriptional activation. The large, central core domain (residues 100–300) contains conserved regions II–V, is involved in DNA binding, and is the location of almost all oncogenic p53 mutations. The basic C-terminal domain (residues 320–360) contains nuclear localization signals and is involved in p53 tetramerization. X-ray diffraction and NMR structures of the core domain and oligomerization domain, respectively, have been determined (19, 20). The core domain is tightly folded and resembles NF-κB in having nine β structures arranged in two sheets. A tetrahedrally coordinated zinc atom holds two loops, L2 and L3, such that a critical Arg residue in L3 contacts bases in the minor groove. A loop-sheet-helix structure contacts bases in the major groove.

As depicted in Fig. 2, the core domain of human p53 has five surface cysteines. If a particular Cys-to-Ser mutation were to result in a p53 protein that no longer required thioredoxin reductase for activity, it would constitute strong evidence that the cysteine normally resident at the site is prone to oxidation and that thioredoxin reductase keeps the cysteine reduced and the protein active. The role of cysteine residues in the regulation of murine p53 activity has been investigated by Rainwater et al. (17). Cys-to-Ser mutations at Cys173 or Cys239 (corresponding to human Cys176 and Cys242) abolish DNA binding, transactivation of reporter gene expression, and tumor suppression. These two cysteines are critical for coordinating zinc and stabilizing the structures of loops L2 and L3. In contrast, Cys-to-Ser mutations at Cys179 or Cys277 (corresponding to human Cys182 and Cys277) have little or no effect on the biological activities of p53, but these mutants were not further tested to see if treatment with reducing agents was still required for enhanced sequence-specific DNA binding of p53.

Evidence for redox control of p53 activity has broad implications. At present, it is not clear how the activity of p53 is regulated. Redox regulation offers a new paradigm for control of transcription factor activity that complements other mechanisms involving phosphorylation (21) and acetylation (22). Reduction of disulfides might be part of the mechanism by which p53 is activated as a transcription factor by DNA damage. Alternatively, oxidation of thiols may be part of the mechanism by which p53 is inactivated once damaged DNA has been repaired and it is appropriate for cells to enter S phase. Evidence suggestive of redox control of transcription factor activity has been obtained for NF-κB (23), Myb (24), AP-1 (25), and PEBP2/CFB (26).

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