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Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA

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ABSTRACT

Redox modulation of wild-type p53 plays a role in sequence-specific DNA binding *in vitro*. Reduction produces a DNA-binding form of the protein while oxidation produces a non-DNA-binding form. Primer extension analysis reveals that increasing concentrations of reduced p53 result in enhanced protection of the consensus sequence, while increasing concentrations of oxidized p53 confer minimal protection of the consensus sequence. DNA binding by oxidized p53 is, therefore, not sequence-specific. In contrast, there is no observable difference in the binding of oxidized p53 and reduced p53 to double-stranded non-specific or mismatched DNA in gel mobility shift assays. Both forms of p53 bind equally well, suggesting that redox modulation of p53 does not play a role in its binding to non-specific or mismatched DNA. In view of the *in vitro* evidence that redox state influences the sequence-specific DNA-binding of p53, we have examined the effect of oxidative stress on the *in vivo* ability of p53 to bind to and transactivate PG_{13}-CAT, a reporter construct containing multiple copies of the p53 consensus binding site linked to the chloramphenicol acetyltransferase gene. Hydrogen peroxide treatment of cells cotransfected with p53 results in a marked decrease in CAT activity, suggesting that oxidation of p53 decreases the ability of the protein to bind to consensus DNA and transactivate target genes *in vivo*.

INTRODUCTION

The tumor suppressor protein p53 is a multifunctional protein implicated in a number of cellular processes: transcriptional regulation of specific target genes (1–4), suppression of cell transformation by oncogenes (5), arrest of cells in response to DNA damage (3), binding to damaged DNA (6,7), reannealing of single-stranded complementary DNA and strand transfer (7–9), inhibition of DNA replication (10,11) and triggering of apoptosis (12,13). The key to p53's involvement in such a diverse array of processes is its ability to bind to DNA. Non-specific, short single-stranded DNA (14), mismatched, 3 nt DNA bulges (6) and a specific double-stranded consensus sequence (15,16), found either in origins of replication (17) or transcriptional regulatory regions of genes transactivated by p53 (1,4,18), are all targets of p53. Some of the parameters which influence the ability of p53 to bind to these various types of DNA have been identified and include different binding domains of the protein molecule, the coordination of a zinc ion by each monomer of p53, and redox modulation of p53.

Site-specific binding of p53 to consensus DNA is dependent upon the highly conserved central domain of the protein (19–21), while binding of p53 to single-stranded DNA, damaged DNA or mismatched DNA is dependent upon the C-terminal region of the protein (6,7). Chelation of the zinc ion, shown by X-ray diffraction studies (22) to be coordinated by amino acids Cys-176, His-179, Cys-238 and Cys-242 of human p53, abolishes sequence-specific DNA binding of p53 (23,24). We have previously shown that individual mutation of the corresponding cysteine residues (Cys-173, Cys-235 and Cys-239) in murine p53 completely blocks transcriptional activation by p53, leads to a striking enhancement rather than suppression by p53 of oncogene-mediated transformation, and markedly decreases sequence-specific DNA binding *in vitro* (24). Redox modulation of p53 plays a role in sequence-specific DNA binding *in vitro*. Nine of the 12 cysteine residues in murine p53 are located in the highly-conserved central DNA-binding domain of p53 (22) and, during oxidation of the protein, would be subject to disulfide bond formation and an altered conformation of the protein. The evidence suggests that reduction of p53 produces an active, specific DNA-binding conformation while oxidation of p53 produces a non-specific DNA-binding conformation of the protein (24–26). However, since a minimal amount of *in vitro* DNA binding of oxidized p53 does occur (24), it was considered important to determine whether this binding was due either to recognition of the consensus sequence by p53 or to non-specific DNA binding by p53. We also wanted to determine if redox modulation of protein structure might play a role in the ability of p53 to bind to non-specific or mismatched DNA *in vitro* and to regulate transcription *in vivo*.

MATERIALS AND METHODS

Purification of wild-type p53 protein

Insect Sf9 cells, growing in Grace's medium supplemented with yeastolate and lactalbumin hydrolysate plus 10% fetal calf serum, were infected with NPVp53, a recombinant baculovirus expressing wild-type murine p53 (27). Extracts were prepared at ~65 h

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post-infection, and p53 protein was obtained by immunoaffinity purification on PAb 421-protein A–Sepharose, as previously described (24). During the process of extraction and purification, the p53 protein is presumed to have undergone oxidation since no effort was made to add fresh dithiothreitol (DTT).

Gel mobility shift assay

Plasmid pBS.KS.Shay DNA was restricted with *Bam*HI and *Eco*RI, and the 61 bp fragment containing the p53 consensus sequence (24) was end-filled with [α-^{32}P]dCTP. In the gel mobility shift assay, purified p53 protein (50–300 ng) was pretreated with freshly-made 5 mM DTT or H_2O for 20 min at room temperature and then incubated with ^{32}P-labeled target DNA (0.5–2 ng) in the presence of either Bluescript or pSV01ΔEP competitor DNA (5–200 ng) for 30 min at room temperature. DNA–protein complexes were analyzed as described previously (24). In assays in which dialyzed protein was used, purified p53 was dialyzed against 20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 10% glycerol at 4°C. When p53 was incubated with antibody, DTT concentration was lowered to 2 mM and an aliquot (500 ng–1 µg) of purified monoclonal PAb 421 or PAb 246 IgG (Calbiochem/Oncogene) was added to oxidized or reduced p53 prior to the addition of labeled target DNA.

DNase I footprinting

Varying concentrations of purified p53 protein were preincubated with 5 mM DTT or H_2O for 20 min at room temperature in DNA binding buffer (25 mM HEPES, pH 7.6, 50 mM KCl, 0.5 mg/ml bovine serum albumin, 20% glycerol, 0.1% NP-40). Supercoiled pBS.KS.Shay DNA (0.5 µg) containing the p53 consensus sequence was added, and the binding reaction proceeded for 20 min at room temperature. DNase I (Worthington DPFF, 0.2 U) was then added to the reaction mixture for 1 min at room temperature. The reaction was stopped by the addition of phenol plus 20 mM EDTA and heated at 80°C for 2 min. The aqueous phase was desalted, and the eluate containing the DNA was analyzed by primer extension (28).

T7 and T3 oligonucleotide primers were end-labeled with [γ-^{32}P]ATP. After alkaline denaturation of the DNase-treated pBS.KS.Shay DNA, the appropriate primer was annealed and extended in the presence of the Klenow fragment (28,29). Aliquots of the DNA samples were analyzed on a 6% urea–formamide gel, along with Sanger dideoxynucleotide sequencing reactions, and the footprint detected by exposure to Kodak XAR-5 film.

CAT transactivation assay

NCI-H358 cells growing on 6 cm diameter plates in RPMI 1640 medium plus 10% fetal bovine serum were doubly transfected with 2 µg PG_{13} CAT (kindly provided by B. Vogelstein) and 2 µg pBS.KS+ plasmid containing wild-type murine p53 cDNA under the control of the Harvey murine sarcoma virus long terminal repeat (27) in the presence of DOTAP liposome transfection reagent (Boehringer Mannheim). Control cells were transfected with 1 µg RSV-CAT (kindly provided by I. Verma) and 3 µg pBS.KS+ DNA. The cells were treated at 16 h post-transfection with hydrogen peroxide (H_2O_2) at final concentrations varying from 50 µM to 1 mM and were harvested at 24 h post-transfection. Lysates were prepared, protein concentrations determined, and CAT assays performed as described previously (24). The ^{14}C-labeled products of the CAT assay were separated by silica gel thin-layer chromatography in chloroform–methanol (19:1) and detected by exposure to Kodak XAR-5 film.

Blotting and immunodetection of p53 expressed in transfected cells

NCI-H358 cells growing on 10 cm diameter plates were doubly transfected with 5 µg PG_{13} CAT and 2 µg pBS.KS+ plasmid containing wild-type murine p53 cDNA under control of the cytomegalovirus (CMV) promoter. Extracts were prepared 24 h post-transfection by lysis in buffer containing 150 mM Tris–HCl (pH 9.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 mg/ml leupeptin and 1 mg/ml aprotinin for 30 min at 4°C. After centrifugation at 18 000 r.p.m. for 20 min at 4°C, aliquots of the supernatant were electrophoresed on a 7.5% SDS–polyacrylamide gel and blotted onto nitrocellulose. The p53 protein on the blot was immunodetected with a mixture of monoclonal antibodies PAb 242, PAb 248 and PAb 421, each diluted 1:500, followed by biotinylated goat anti-mouse immunoglobulin G and streptavidin–alkaline phosphatase (Immunoselect system, Gibco/BRL) and visualized with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) and Nitro Blue Tetrazolium (NBT).

RESULTS

Increasing concentrations of protein override the effect of oxidation–reduction on binding of p53 to DNA in mobility shift assays

We and others have previously shown that binding of purified p53 to a DNA fragment containing a specific consensus sequence is markedly stimulated by reduction of the protein with dithiothreitol (DTT) (24–26). In addition, DTT treatment of p53 protein affects the electrophoretic mobility of the p53–DNA complexes, such that the complexes containing oxidized p53 migrate more rapidly than the complexes containing reduced p53 (24). This difference in migration is thought to be due to an altered migration of the protein rather than of the nucleoprotein complex, since a similar difference is seen when analyzing the migration of oxidized versus reduced p53 protein in the absence of DNA (24, and data not shown). Since a minimal amount of oxidized p53 does in fact bind to consensus DNA and cause a mobility shift, we wanted to examine the effect of increasing concentrations of protein on DNA binding.

Surprisingly, a 3-fold increase in p53 concentration resulted in almost as much binding of target DNA by oxidized p53 as by reduced p53 (Fig. 1). The results suggest that it might be possible to override the redox control of p53 binding to DNA in a quantitative manner, by altering the ratio of protein to DNA. However, we decided to examine the site-specific binding of p53 in a more definitive way, by DNase footprinting, since the migration of complexes containing oxidized p53 was clearly different from the migration of those containing reduced p53 (Fig. 1), and it was not clear that protection of the consensus sequence would be the same in both cases.

DNase footprinting reveals that DNA binding by oxidized p53, even at high protein:DNA concentrations, is not site-specific

DNase footprints using primer extension analysis (28) were obtained to define more precisely the interaction between oxidized

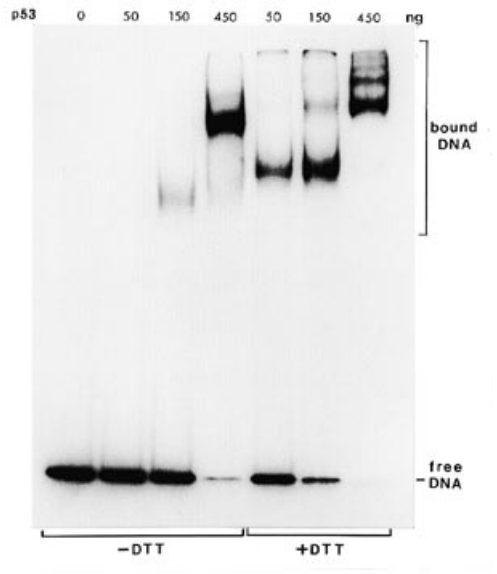

Figure 1. Effect of protein concentration on binding of oxidized versus reduced p53 to sequence-specific DNA. Purified p53 protein (50–450 ng) was either treated with H_2O (oxidized) or reduced with 5 mM DTT prior to incubation with 2 ng ^{32}P-labeled consensus DNA plus 20 ng unlabeled Bluescript SK$^+$ DNA. Protein–DNA complexes were separated from unbound DNA by gel electrophoresis and visualized by autoradiography.

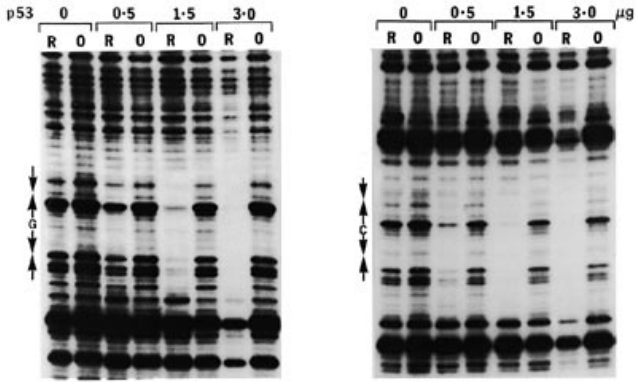

Figure 2. Protection of the consensus sequence by reduced, but not oxidized, p53 bound to supercoiled DNA in primer extension DNase footprints. Varying concentrations (0.5–3.0 µg) of purified p53 were preincubated with 5 mM DTT or H_2O prior to the addition of 0.5 µg supercoiled plasmid DNA (pBS.KS$^+$) containing the consensus sequence and then allowed to bind for 20 min prior to DNase I treatment. ^{32}P-end-labeled T7 (left panel) or T3 (right panel) primers were annealed to the DNA, extended with Klenow, and analyzed by denaturing gel electrophoresis. R, reduced p53 protein; O, oxidized p53 protein. On a molar basis, 1.5 µg of p53 incubated with 0.5 µg pBS.KS$^+$ DNA for the DNase footprint is equivalent to 300 ng of p53 incubated with 2 ng of pBS.KS.Shay consensus DNA fragment in the gel shift assay (Fig. 1).

p53 or reduced p53 and supercoiled DNA containing the consensus sequence of Funk *et al.* (16). The ratios of purified p53 protein to target DNA were similar to those used in the gel mobility shift assays.

Primer extension analysis showed that increasing concentrations of reduced wild-type p53 protein result in increased protection of the consensus sequence, while increasing concentrations of oxidized wild-type p53 protein confer minimal protection (Fig. 2). It should be noted that supercoiled Bluescript DNA containing the consensus sequence was used for the DNase footprints, whereas a linear 61 bp DNA fragment containing the consensus sequence along with a 10-fold excess of competitor Bluescript DNA (without the consensus sequence) was used in the mobility shift assays. Similar results were obtained with standard DNase footprints, in which one strand or the other of the linear 61 bp fragment was end-labeled with [γ-^{32}P]phosphate and T4 polynucleotide kinase. Reduced p53 protected the consensus sequence, whereas oxidized p53 did not (data not shown). The results obtained with both methods suggest that the binding of oxidized p53 to DNA that is observed in the mobility shift assay must be random and non-specific, no matter what ratio of protein to DNA is used.

It is interesting to note that one of the two DNA strands is preferentially protected by reduced p53 in the primer extension assays (Fig. 2), suggesting that p53 binds to one side of the helix as originally noted in both the X-ray crystallography studies of Cho *et al.* (22) and the gel mobility shift assays of Wang *et al.* (30). The functional significance of this observation is not known.

Oxidized p53 binds as well as reduced p53 to double-stranded non-specific or mismatched DNA

It has recently been shown that p53 binds preferentially to double-stranded damaged DNA (7) and to mismatched DNA, at the site of the mismatch (6). In view of the observed differential binding of oxidized p53 and reduced p53 to consensus DNA (Figs 1 and 2), we wished to determine if there were similar differences in the binding of oxidized and reduced p53 to mismatched DNA and to non-specific DNA. The non-specific DNA used was a double-stranded 49mer (6), while the mismatched DNA was the identical 49mer containing an insert in the center of the DNA of either three adjacent C-residues (3C) or three copies of three adjacent C-residues (3-3C) in one strand but not the other (initially provided by Jack Griffith). In marked contrast to the differential binding of p53 to sequence-specific DNA (Fig. 3), there was no obvious difference in the binding of oxidized p53 and reduced p53 to either non-specific DNA (Fig. 3) or mismatched DNA (Fig. 4), even at varying protein concentrations. Addition of increasing amounts of competitor DNA does not alter the equal binding of oxidized and reduced p53 to non-specific or mismatched DNA (data not shown).

It appears, therefore, that redox modulation of p53 does not play a role in binding of the protein to double-stranded non-specific or mismatched DNA. This finding is understandable in view of the fact that binding to such DNAs has been shown to be dependent on the C-terminus of p53 (6,7) while the portion of p53 known to be subject to redox modulation is the central domain, responsible for sequence-specific DNA binding (22).

Incubation of p53 with PAb 421, but not with PAb 246, inhibits the binding of oxidized p53 but not binding of reduced p53 to target DNAs

Additional evidence for a conformational difference between oxidized and reduced p53 was obtained from studies using monoclonal antibodies PAb 421 and PAb 246. PAb 421 recognizes an epitope at the C-terminus of p53, encompassing amino acids 363–372, whereas PAb 246 recognizes an epitope located on the N-terminal side of the central DNA-binding domain, spanning amino acids 86–107 (31). A striking difference between oxidized and reduced p53 was noted when purified protein was incubated

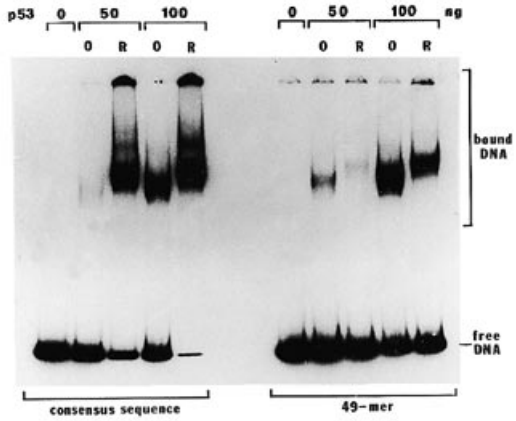

Figure 3. Comparison of binding of oxidized and reduced p53 to sequence-specific DNA versus non-specific DNA. Varying concentrations of oxidized or reduced p53 (50–100 ng) were incubated with 1 ng of either ^{32}P-labeled DNA containing the consensus sequence or ^{32}P-labeled non-specific DNA (49mer) plus 10 ng unlabeled Bluescript SK$^+$ DNA. O, oxidized p53 protein; R, reduced p53 protein.

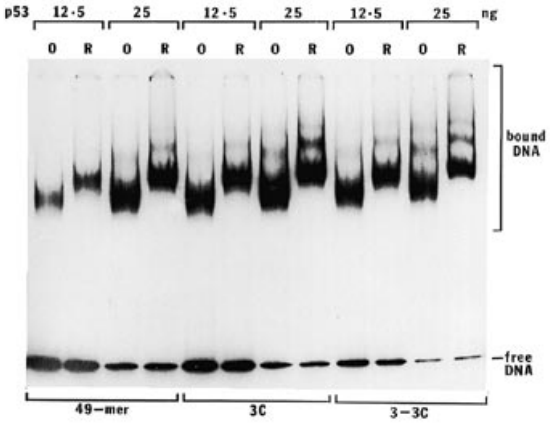

Figure 4. Equal binding of oxidized and reduced p53 to double-stranded, non-specific or mismatched DNA. Oxidized or reduced p53 (12.5–25 ng) was incubated with 0.5 ng ^{32}P-end-labeled 49 bp non-specific DNA containing either no mismatch (49mer), a single three-cytosine bulge at the center (3C) of one strand, or three three-cytosine bulges at the center (3-3C) of one strand, in the presence of 5 ng unlabeled pSV01ΔEP DNA, and then electrophoresed on a mobility shift gel. O, oxidized p53 protein; R, reduced p53 protein.

with PAb 421 IgG, but not with PAb 246 IgG, prior to incubation with target DNA.

Incubation with PAb 246 IgG supershifts both oxidized and reduced p53 bound to double-stranded 61 bp DNA containing the consensus sequence (Fig. 5). In contrast, incubation with PAb 421 IgG inhibits the binding of oxidized p53 to the consensus DNA, but supershifts reduced p53 bound to the same DNA (Fig. 5) in a manner similar to that previously reported by Hupp *et al.* (32). The data suggest that binding of oxidized p53 to the consensus DNA is dependent on the C-terminus of the protein, whereas binding of reduced p53 is not. These findings are in agreement with the conclusion drawn from the DNase footprints, that binding of oxidized p53 to DNA containing the consensus

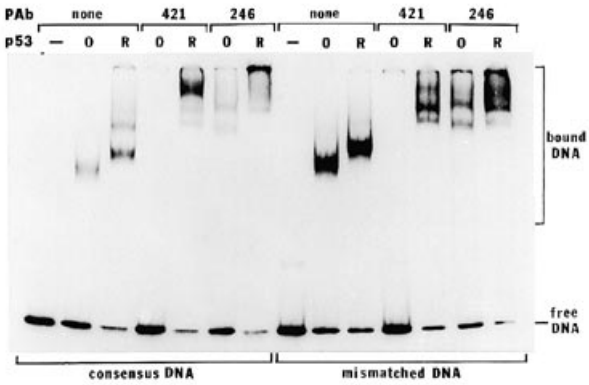

Figure 5. Effect of monoclonal antibody PAb 421 versus PAb 246 on the binding of oxidized versus reduced p53 to target DNA. Purified p53 protein (50 ng) was either untreated (oxidized) or pretreated with 2 mM DTT (reduced) for 15 min prior to the addition of PAb 421 or PAb 246 for 10 min incubation at room temperature. The protein plus antibody mixtures were then incubated with ^{32}P-end-labeled consensus DNA (0.5 ng) or mismatched 3C DNA (0.5 ng) in the presence of unlabeled pSV01ΔEP DNA (5 ng) for 30 min at room temperature, before analysis by mobility shift gel electrophoresis. O, oxidized p53 protein; R, reduced p53 protein.

sequence is non-specific in nature. Others have previously demonstrated that non-specific DNA-binding of p53 is dependent on the C-terminus (6,7).

To our surprise, similar results were obtained when oxidized and reduced p53 were incubated with the same monoclonal antibodies prior to incubation with mismatched DNA. PAb 421 IgG inhibited the binding of oxidized p53, but not that of reduced p53, to the mismatched 3C DNA (Fig. 5), and PAb 246 IgG supershifted both oxidized and reduced p53 bound to the same DNA (Fig. 5). This suggests that, even though oxidized and reduced p53 bind equally to mismatched DNA, the mechanism of binding of the two forms of the protein to this target DNA must differ, with the binding of oxidized p53, but not that of reduced p53, being solely dependent on the C-terminus.

Oxidation of cells by hydrogen peroxide decreases transactivation by p53

In view of the *in vitro* evidence suggesting that redox state influences the sequence-specific DNA-binding of p53, we proceeded to determine if oxidative stress might also affect the ability of p53 to bind to and transactivate specific target sequences within cells. To examine the binding of p53 to target DNA *in vivo*, we cotransfected NCI-H358 cells that have no endogenous p53 function (24) with DNA expressing wild-type p53 and with PG_{13}-CAT DNA, a construct containing 13 copies of a p53 consensus DNA-binding sequence linked to the chloramphenicol acetyltransferase (CAT) gene (33). At 16 h after transfection, 50 μM–1 mM H_2O_2 was added to the growth medium, and the cells were inspected every 2 h for changes in morphology. The cells were harvested 8 h later, at 24 h post-transfection, and CAT assays were performed after equalizing the protein concentrations of the extracts.

When increasing concentrations of hydrogen peroxide were used to treat the transfected cells, there was a marked decrease in CAT activity, as indicated by the decrease in conversion of [^{14}C]-chloramphenicol to [^{14}C]monoacetyl-chloramphenicol (Fig. 6A). It

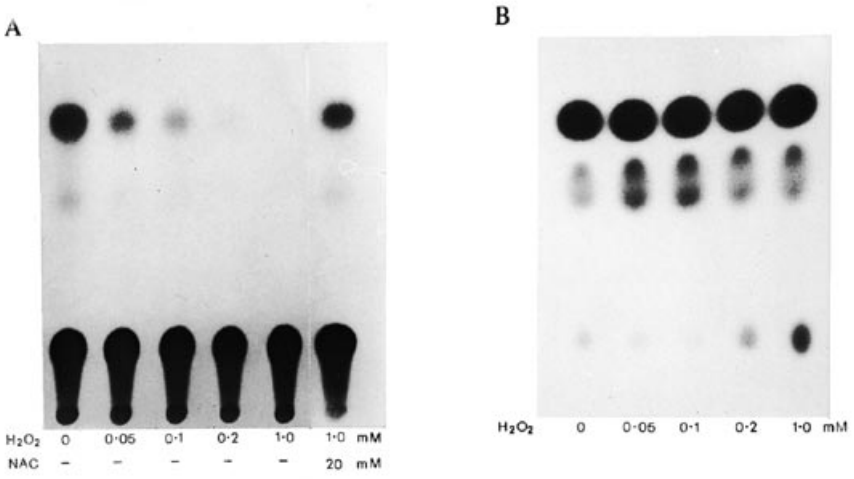

Figure 6. The effect of hydrogen peroxide treatment on transactivation in H358 cells transfected with either PG_{13}-CAT DNA + wild-type p53 DNA (**A**) or, as a control, with RSV-CAT DNA (**B**). Transiently transfected cells were treated with varying concentrations of H_2O_2 (50 μM–1 mM) for 8 h prior to preparation of cell extracts for the determination of CAT activity. In one case, cells were treated simultaneously with 1 mM H_2O_2 and 20 mM *N*-acetylcysteine, an anti-oxidant. The conversion of [^{14}C]chloramphenicol to [^{14}C]mono-acetyl-chloramphenicol by CAT was determined through separation of the products by silica thin-layer chromatography, with [^{14}C]mono-acetylchloramphenicol migrating farther from the origin.

is interesting to note that when 20 mM *N*-acetylcysteine (NAC) was added simultaneously with 1 mM H_2O_2 to the growth medium of the transfected cells, the amount of CAT activity was somewhat greater than that seen when the cells were treated with 50 μM H_2O_2 alone (Fig. 6A). The protective effect of NAC, a known anti-oxidant, is presumably due to its ability to increase depleted glutathione levels in cells and thereby to reduce the reactive oxygen species produced by hydrogen peroxide treatment. The data suggest that the oxidized state of the cells induced by hydrogen peroxide affects the ability of p53 to bind in a site-specific manner to target DNA, presumably by altering the conformation of the protein, although an effect of oxidation on other proteins involved in transactivation cannot be formally excluded.

To determine if the decrease in transactivation ability of p53 was due to a non-specific toxic effect of hydrogen peroxide on the cells, we transfected RSV-CAT DNA into NCI-H358 cells and tested the effect of increasing concentrations of hydrogen peroxide on the expression of CAT from the constitutively-transactivated promoter (Fig. 6B). In contrast to transactivation of PG_{13}-CAT by p53, which was sensitive to 50 μM H_2O_2, transcription of RSV-CAT only appeared to be affected by H_2O_2 at a concentration of 1 mM, providing evidence for the specificity of the effect on p53.

Immunoblotting analysis of extracts from NCI-H358 cells transfected with p53 DNA under control of the CMV promoter showed similar, or somewhat increased, levels of p53 protein in cells treated with varying concentrations of hydrogen peroxide (Fig. 7), indicating that the decrease in CAT activity was not due to a decrease in p53 protein in response to the H_2O_2 treatment. The increased level of p53 seen in cells treated with 1 mM H_2O_2 may or may not be significant, because this concentration of H_2O_2 decreases the total amount of cellular protein and therefore p53 represents a higher percentage of the total protein loaded on the gel.

There was a marked effect of hydrogen peroxide on the morphology of the NCI-H358 cells, as revealed by microscopic examination every 2 h after addition of H_2O_2 to the culture

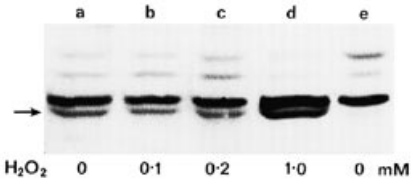

Figure 7. Immunoblot analysis of p53 protein in extracts from untransfected H358 cells or cells transfected with wild-type p53 DNA and subjected to varying concentrations of hydrogen peroxide. Extracts from H358 cells transfected with wild-type 53 DNA (lanes a–d) and treated with varying concentrations of H_2O_2 (100 μM–1 mM) were compared with extract from untransfected H358 cells (lane e) not treated with H_2O_2. Proteins were separated by SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose and p53 detected with a mixture of monoclonal antibodies PAb 242, PAb 248 and PAb 421, followed by biotinylated secondary antibody and streptavidin–alkaline phosphatase. The p53 in the immunoblot is indicated by an arrow. The slower-migrating protein detected non-specifically is not p53-related, since it is present in untransfected as well as transfected H358 extracts.

medium. After 7 h of treatment, those cells treated with either 100 or 200 μM H_2O_2 were somewhat rounder than the control untreated cells, whereas those cells treated with 1 mM H_2O_2 were either very round or else detached and floating, with heavy membrane blebbing. In fact, membrane blebbing was observed within 1 h after initial treatment of the cells with 1 mM H_2O_2. Preliminary results using fluorescein-conjugated annexin V (R & D Systems) to detect apoptotic cells suggest that the cells with altered morphology are undergoing apoptosis.

DISCUSSION

Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA

It appears that p53 belongs to a growing list of transcriptional activators which are subject to redox modulation (34). We have previously shown that redox regulation of p53 involves two

clusters of cysteine residues in the central domain of the protein (24). One cluster contains three cysteines (residues 173, 235 and 239) responsible for the coordination of a zinc ion by each p53 monomer (22). Mutation of any one of these three cysteines results in nearly a complete loss of binding of p53 to sequence-specific DNA, loss of transactivator function and enhancement, rather than suppression, of cell transformation by p53 (24). The other cluster of cysteines (residues 121, 132, 138 and 272) is located in or near the loop–sheet–helix region of p53 that makes contact with the consensus DNA sequence. This cluster is thought to account for a second level of redox regulation that does not involve the interaction of p53 with zinc (24). It is presumably the redox state of these two cysteine clusters that is critical for the functioning of p53 as a transcriptional regulator.

Both our *in vitro* and *in vivo* data suggest that p53 must be in a reduced state in order to bind to specific consensus DNA and subsequently control transcription of adjacent genes. This conclusion fits well with the fact that Ref-1 has recently been found to stimulate DNA binding and transactivation by p53 *in vitro* (35). Ref-1 is an interesting bifunctional protein, responsible for reduction of a critical cysteine residue required for DNA binding in both fos and jun (36), as well as being responsible for endonuclease activity in repair of DNA lesions caused by oxidative damage (37,38). It appears to play an important role in protection of cells against DNA damaging agents and against changes in oxygen tension, whether by hypoxia or hyperoxia (39,40).

Wild-type p53 has been shown to bind to mismatched DNA at the site of the mismatch (6), suggesting that it is involved either directly or indirectly in the process of DNA repair in cells which have undergone genotoxic damage. Our finding that oxidized and reduced p53 bind equally well to mismatched DNA suggests that, in contrast to a marked effect on transcriptional regulation by p53, the redox state of the cell does not affect the potential role of p53 in mismatch repair. The differential versus non-differential binding of p53 to DNA and factors that influence the redox state of p53 are summarized in Figure 8. The apparent lack of binding of oxidized p53 to sequence-specific DNA suggests that oxidized p53 is not able to transactivate target genes known to be regulated by wild-type p53 *in vivo*. However, the possibility remains that oxidized p53 might bind to DNA that differs from the p53-recognized consensus sequences isolated to date and be responsible for transactivation of a unique set of genes.

The differential effect of PAb 421 on binding of oxidized versus reduced p53 to mismatched DNA indicates that more than one domain of the reduced protein, but not of the oxidized protein, is capable of binding to DNA non-specifically. The complete inhibition of binding by PAb 421 argues that oxidized p53 can bind non-specifically to DNA only through its C-terminal domain. In contrast, supershifting of the reduced p53–DNA complex by PAb 421 suggests that there is a domain in addition to the C-terminal domain that is responsible for the non-specific DNA binding of reduced p53. It seems reasonable to assume that the central domain of p53 is capable of non-specific as well as specific DNA binding and that the ability of this domain to function in both types of DNA-binding activity is dependent on the reduction of critical cysteine residues located in the central domain (24). This domain is presumably hidden and non-functional in oxidized p53 as a result of an altered conformation of the protein. Possibly interaction of a cellular protein with oxidized p53 at its C-terminus alters the binding of oxidized p53 to mismatched DNA *in vivo*, while not affecting the binding of reduced p53.

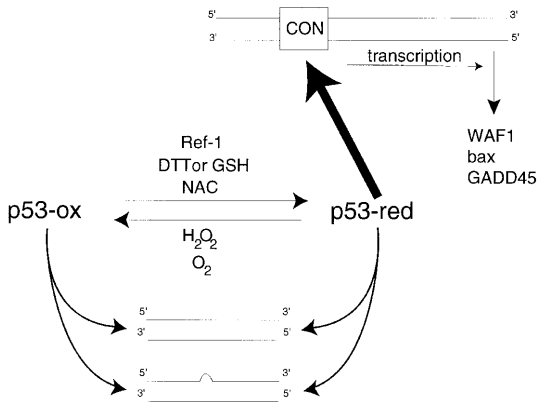

Figure 8. Summary of the role of the redox state of p53 on binding of the protein to DNA. There is differential binding of oxidized and reduced p53 to sequence-specific DNA (CON), as opposed to non-differential binding to non-specific or mismatched DNA.

Transcriptional regulation by p53 is altered by treatment of cells with the oxidant hydrogen peroxide

Perturbation of cells by environmental agents such as ionizing radiation or hydrogen peroxide (H_2O_2) induces an increase in the intracellular level of reactive oxygen intermediates (ROIs). An above-normal level of ROIs, referred to as oxidative stress, can cause genotoxic damage to the cell. In order to avoid DNA damage, it appears that both prokaryotic and eukaryotic cells respond to oxidative stress by means of specific transcriptional regulatory factors which are themselves subject to redox regulation.

Prokaryotic cells have been shown to initiate oxidative stress responses via the transcription factors OxyR and SoxR. SoxR responds to O_2^- by changing the redox state of its Fe-S cluster, going from an inactive to an active transcription factor (41). Transcriptional regulation by OxyR is somewhat more complex, in that it can function as both an activator and as a repressor, regulating different promoters under oxidizing versus reducing conditions through differential DNA binding forms (42).

Some oxidative stress response factors have been identified in eukaryotic cells. NF-κB, when activated by H_2O_2, binds to DNA and initiates transcription, whereas treatment of cells with NAC prevents the activation of NF-κB by H_2O_2 (43). Similarly, both UV and H_2O_2 rapidly induce a lasting increase in AP1 binding activity *in vivo* (44). Contradictory evidence, however, indicates that AP1 is only weakly responsive to H_2O_2 and that it is activated when cells are treated with the reducing agent PDTC (pyrrolidine dithiocarbamate) or are subjected to transient expression of thioredoxin, which induces an anti-oxidant state (45). This latter data agrees with the marked enhancement of *in vitro* DNA binding observed when fos and jun are reduced and with their lack of binding to DNA when oxidized (46).

We propose that p53 also belongs to the family of oxidative stress response factors found within eukaryotic cells. Exposure of cells to hypoxia induces the accumulation of both p53 (47) and Ref-1 (48), a protein known to maintain the reduced state of cysteine residues required for DNA binding (46). The hypoxia-induced increase in p53 and Ref-1 may have functional signifi-

cance *in vivo* since a significantly higher frequency of apoptosis has been found in hypoxic regions of tumors containing wild-type p53 compared to hypoxic regions of p53-negative tumors (49), and transactivation by p53 is required for induction of apoptosis (4,49). Others have shown that the genotoxic agents UV and ionizing radiation, known to generate highly damaging reactive oxygen species, trigger an increase in the level of p53 within targeted cells (50,51). This response appears to be regulated post-transcriptionally (51,52), although others have shown that after treatment of cells with anticancer drugs there is enhanced transcription of p53 dependent on a core promoter element of the p53 gene (53). In general, it is agreed that the increase in p53 protein results in enhanced transcription of target genes, such as WAF1 (54) and bax (4), whose gene products are required for G_1 cell cycle arrest and induction of apoptosis respectively in response to DNA damage. Our data suggest that an increase in p53 protein is not in and of itself sufficient for increased transcriptional regulation, but that in addition the protein must be in a reduced conformation to act as a transcriptional regulator within cells that have undergone genotoxic damage.

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