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Direct redox modulation of p53 protein: potential sources of redox control and potential outcomes

Review Article

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Abbreviations: ROI, reactive oxygen intermediates; **Ref-1**, Redox factor-1; **DTT**, dithiothreitol; **ESR**, electron spin resonance; **PDTC**, pyrrolidine dithiocarbamate; **GSH**, glutathione; **SOD**, superoxide dismutase; **TPEN**, N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine

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Summary

Appropriate response to environmental stressors is essential for life. Many stressors, such as UV light, ionizing radiation, reactive oxygen intermediates (ROI), heat shock and hypoxia alter the redox potential of the cell. Recently, it has been shown that some of these stressors promote direct oxidation of specific protein cysteine residues resulting in either up-regulation or down-regulation of protein activity in the cytosol. In higher eukaryotes, the *p53* tumor suppressor gene is a central component of stress response and its activation results in either cell cycle arrest or apoptosis. In cultured cells, p53 appears to become activated by some stressors (hydrogen peroxide, heat) predicted to directly increase cellular redox potential. However, *in vitro* studies indicate that p53 protein oxidation inhibits its ability to bind its consensus sequence DNA. If p53 is unable to bind consensus sequence DNA, p53 is predicted to be incapable of activating the $p21^{WAF1/CIP1}$ gene, responsible for mediating G1 cell cycle arrest. Two proteins previously shown to reduce oxidized cytoplasmic proteins, Redox factor-1 and thioredoxin reductase, have been shown to play important roles in maintaining p53 activity, suggesting that they may be responsible for keeping p53 in the reduced state inside the cell. Analysis of the p53 crystal structure revealed several well-conserved cysteine residues exposed on the protein surface that may be susceptible to oxidation. Based on this analysis we predict that cysteine residues 124, 176, 182, 242 and 277 are primary candidates for redox regulation. In this communication, we review the data demonstrating p53 regulation by direct alteration of p53 cysteine residue oxidation, propose a testable mechanism by which p53 oxidation may occur, and discuss the possible implications of p53 oxidation on cell growth control and DNA repair.

I. Introduction

The *p53* tumor suppressor gene is one of the most frequently mutated genes in human cancers (Baker et al., 1989; Nigro et al., 1989; Hainaut et al., 1998). It is a cell cycle checkpoint gene responsible for committing mammalian cells to a growth arrest phenotype or apoptosis in response to genotoxic and non-genotoxic stressors (Levine, 1997; Giaccia and Kastan, 1998). The *p53* gene encodes a transcription factor that is synthesized in a latent form and can be activated by a wide range cell stressors. Once activated, the intracellular p53 protein level increases and p53 binds, in sequence-specific fashion, to certain DNA promoters which, in turn, leads to activation of genes that mediate cell cycle arrest (El-Deiry et al., 1993; Chin et al., 1997; Hermeking et al., 1997; Bunz et al., 1998) or apoptosis (Miyashta and Reed, 1995). One of the p53 responsive genes that appears necessary for mediating G1 arrest in several cell types is $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor (El-Deiry et al., 1993; Harper et al., 1993). Activation of p53 is complex and inhibition of this process can lead to loss of cell growth control.

Activation of p53 is thought to take place at both the translational and post-translational level. Most recent work has concentrated on understanding the post-translational events that lead to p53 activation. The exact activation pathway is highly dependent on the type of stressor applied. Each class of stressors appears to result in a unique pattern of p53 protein phosphorylation and acetylation to achieve p53-mediated transcription of appropriate downstream targets (Giaccia and Kastan, 1998). Concomitant with these modifications p53 protein levels increase. Another potential post-translational modification system less extensively explored is direct p53 redox regulation. Protein redox alterations in response to environmental agents, was proposed to occur more than four decades ago (Barron, 1951), but it is only within the past 5 years that, with the advent of new techniques, solid evidence has accumulated indicating that redox changes can occur on cytoplasmic proteins *in vivo* (Åslund and Beckwith, 1999). **Table 1** lists a partial set of cytoplasmic proteins that have been shown to be oxidized at cysteine residues in cells and the effect of oxidation on their activities. For illustrative purposes, a few of these will be discussed.

Three types of chemical oxidation have been identified on protein cysteine sulfhydryl groups in cells. The first type of oxidation is intramolecular disulfide bond formation. This was shown to occur on two bacterial proteins, OxyR and Hsp33 (Zheng et al., 1998; Jakob et al., 1999). Treatment with hydrogen peroxide or heat leads to the formation of intramolecular disulfide bonds and results in the activation of the protein as a transcription factor, in the case of OxyR, or a chaperone protein, in the case of Hsp33. Oxidized OxyR transactivates a panel of genes responsible for protecting the organism from hydrogen peroxide poisoning including hydroperoxidase I, alkyl hydroperoxidase reductase and glutathione reductase (Jamieson and Storz, 1997). Oxidized Hsp33 protects enzymes from denaturing during heat stress or hydrogen peroxide treatment (Jakob et al., 1999).

Table 1. Cytoplasmic proteins oxidized *in vivo* and the effect of oxidation on protein function.

Protein	Species	Protein Function	Oxidizer	Cysteine adduct	Result of oxidation	Reference
OxyR	*E. coli*	Transcription factor	H_2O_2	Intramolecular disulfide bond	Activation, leads to expression of H_2O_2 defense genes	Zheng et al., 1998
Hsp33	*E. coli*	Chaperone	heat/H_2O_2	Intramolecular disulfide bond	Activation, leads to protection of other enzymes susceptible to denaturation	Jakob et al., 1999
SoxR	*E. coli*	Transcription factor	$O_2^{\cdot-}$, NO	One-electron oxidation or assembly of Fe-S centers	Activation, leads to expression of $O_2^{\cdot-}$ defense genes	Demple, 1999 Hidalgo et al., 1997
p53	cultured rat cells	Transcription factor	PDTC[3]	Cysteine residue oxidation-unknown adduct	Inactivation, prevents p53-mediated upregulation of MDM2, $p21^{WAF1/CIP1}$	Wu and Momand, 1998 Verhaegh et al., 1997
phospha-tase 1B	cultured human cells	Dephospho-rylase	EGF	Cysteine residue oxidation-unknown adduct	Inactivation, leads to net Tyr residue phosphorylation of EGFR and activation of the EGF pathway	Lee et al., 1998
GAPDH	human monocytes	Conversion of GAP[1] to 1,3-DPG[2]	H_2O_2	Cysteine residue *S*-thiolation, unknown adduct	Reversible inactivation	Ravichandran et al., 1994
Cardiac creatine kinase	cultured bovine, rat heart cells	Phosphorylate creatine	diamide	Cysteine residue *S*-thiolation-cysteine or glutathione	Partial inactivation of kinase activity	Collison and Thomas, 1987
Tdh3	*S. cerevestae*	Conversion of GAP to 1,3-DPG	H_2O_2	Cysteine residue *S*-thiolation unknown adduct	Reversible inactivation	Grant et al., 1999

[1] Glyceraldehyde 3-phosphate

[2] 1,3-diphosphoglycerate

[3] Pyrrolidine dithiocarbamate

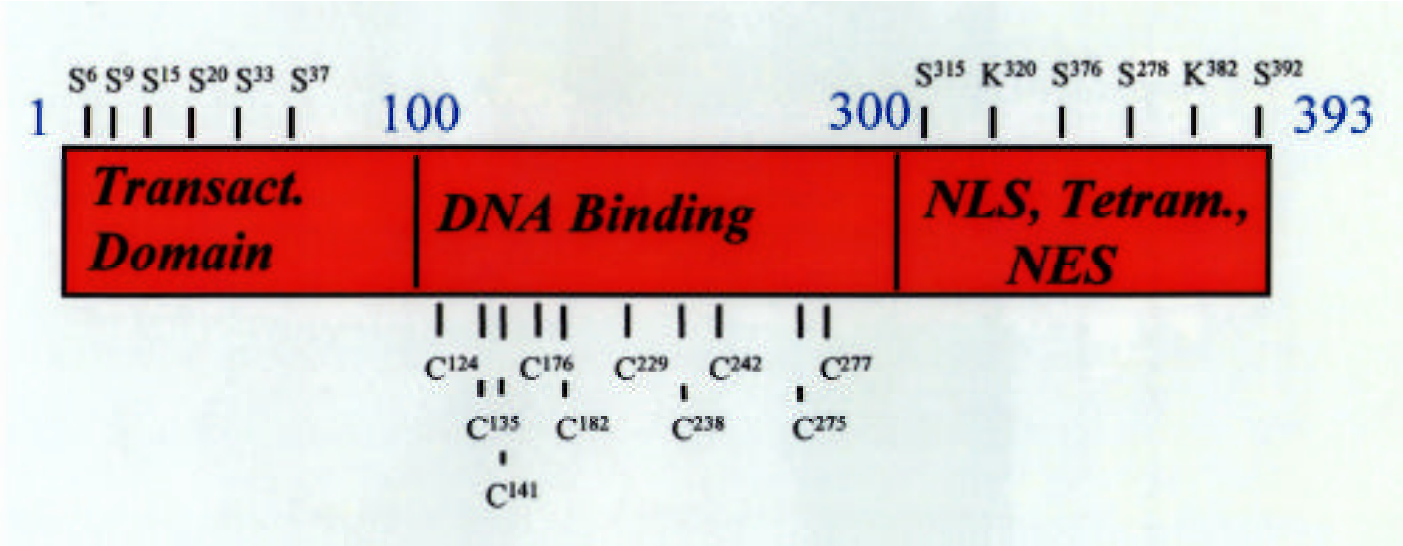

Figure 1. **Schematic diagram of three basic p53 protein functional domains in human p53.** Primary protein sequence of p53. The numbered serine (S) and lysine (K) residues are sites of phosphorylation and acetylation respectively. All cysteine residue positions are shown.

The second type of chemical oxidation involves the oxidation of a metal-sulfur center active site. The bacterial transcription factor SoxR falls into this category (Ding et al., 1996). Like OxyR, SoxR activates genes responsible for bacterial oxidation defense. Target genes upregulated by SoxR include Mn-containing superoxide dismutase, glucose-6-phosphate dehydrogenase and the DNA repair enzyme endonuclease IV. Aside from intramolecular disulfide bond formation and oxidation of metal sulfur centers, a third type of chemical oxidation that occurs in proteins is a disulfide bond formed between a protein cysteine residue and a small molecular weight thiol molecule. Some enzymes have been shown to form a disulfide bond with glutathione (Thomas et al., 1994; Cabiscol and Levine, 1996) and cysteine (Sato et al., 1996). Recently, it was shown that p53 is oxidized in cultured cells in the presence of a metal chelator/oxidant, called pyrrolidine dithiocarbamate (PDTC) (Wu and Momand, 1998).

Oxidation of p53 correlated with inhibition of its transactivation activity. The chemical nature of the p53 cysteine residue oxidation is not known nor, aside from PDTC, are the types of other stressors that lead to p53 oxidation. In this review, we hope to shed light on the possible reactive cysteine residues within p53 and the role direct redox regulation plays in modulating this protein's function.

To understand how oxidation might affect p53 function it is important to review, briefly, the location of the different functional domains of p53 in relation to the p53 cysteine residues (for extensive reviews on this subject see Gottlieb, 1996; Greenblatt, 1994). As shown in **Fig. 1**, the p53 protein can be divided roughly into three distinct domains based on function. All regulatory post-translational modifications, thus far, have been shown to occur in the N- and C-terminal domains. The N-terminal 42 amino acid residues is required for p53-mediated transactivation. For p53 to mediate transcription, this domain must bind hTAFII31 and hTAFII70 accessory transcription factors that form part of the TATA box binding protein complex TFIID. Six serine residues within the N-terminal domain can be phosphorylated. Phosphorylation at Ser 15 is required, in some instances, to upregulate p53 protein levels (Shieh et al., 1997). The C-terminal domain (301-393) includes the tetramerization domain, the nuclear localization sequence and a nuclear export sequence. There are four serine residues regulated by phosphorylation and two lysine residues regulated by acetylation in the C-terminal domain. The central domain of p53 (residues 100-300) can bind DNA in a sequence-dependent manner at two palindromic half-sites with the sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'. Mutations in p53 observed in human tumors and malignancies almost always map to this central DNA binding domain. Proteins with missense mutations in this domain are usually incapable of binding p53 consensus sequence containing DNA elements. All conserved cysteine residues are observed in this domain and, in some cases, Cys residues are mutated in cancers. The frequency of the mutations is fairly low and therefore, cysteine residues are not considered as mutation hot spots (Sun and Oberley, 1996). Direct redox changes on p53 itself would likely affect one or more of these cysteine residues. Given their close proximity to the DNA binding domain it is likely that oxidation of some critical cysteine residues will affect sequence-specific binding activity of p53.

II. p53 activity is upregulated in response to agents that increase intracellular reactive oxygen intermediates

Protein disulfide formation can occur in the cytosol when intracellular reactive oxygen intermediates (ROI) are created (Halliwell and Gutteridge, 1989). ROI have long been thought to be part of the multitude of small intracellular molecules that signal specific transduction pathways within the cell. The three common types of intracellular ROI postulated to be important for modulating protein redox levels are hydrogen peroxide, hydroxyl radicals and superoxide. Specific enzymes have evolved to rid the cell of hydrogen peroxide and superoxide and these enzymes are, in fact, regulated in response to these molecules. Intracellular peroxides are generated at sites of inflammation by secretion of hydrogen peroxide by neutrophils (Vile et al., 1998). Hydroxyl radicals are generated in cells in response to UV light, ionizing radiation and free metals. Interestingly, doxorubicin, a common chemotherapeutic agent used in the treatment of tumors, also appears to generate hydroxyl radicals, that may contribute to its anti-neoplastic properties (Doroshaw, 1986).

Both genetic and cell biology studies suggest that p53 can be activated by stimuli that also result in intracellular ROI production. Solar UV radiation leads to cellular p53 protein elevation and agents that scavenge hydroxyl radicals prevent p53 protein elevation (Vile, 1997). In cultured normal human fibroblasts, treatment with Cd, a metal known to catalyze the formation of hydroxyl radicals, or hydrogen peroxide leads to accumulation of p53 in the nucleus, a sign of p53 activation in some cells (Sugano et al., 1995; Uberti et al., 1999). In one study, normal human fibroblasts treated with a sublethal dose of hydrogen peroxide underwent long term growth arrest, suggestive of p53 activation (Chen et al., 1998). In IMR-90 fetal lung cells, p53 and $p21^{WAF1/CIP1}$ protein levels were transiently elevated in response to hydrogen peroxide (Chen et al., 1998). Hydrogen peroxide-mediated upregulation of p53 protein was inhibited by the iron chelator deferoxamine, suggesting that intracellular hydroxyl radical formation, perhaps generated by Fenton-type chemical reactions, is an important component of this signaling pathway. When the viral oncoprotein E6, a p53-inhibiting protein, was expressed in IMR-90 cells, H_2O_2 treatment failed to upregulate p53 levels or to induce G1 arrest and there was a diminution in the level of $p21^{WAF1/CIP1}$ increase. These studies suggest that H_2O_2 and most likely hydroxyl radicals are important intracellular molecules that lead to p53 activation and cell cycle arrest.

Increases in intracellular ROI levels can also lead to p53-dependent programmed cell death. Using murine embryo fibroblasts cells derived from p53 -/- mice, it was shown that hydrogen peroxide leads to p53-dependent cell death (Yin et al., 1998). Similarly, normal human fibroblasts engineered to express E6 failed to undergo programmed cell death while normal human fibroblasts without E6 underwent programmed cell death in response to hydrogen peroxide suggesting that p53 is required for cell death (Yin et al., 1999). In summary, agents known to increase intracellular hydrogen peroxide or hydroxyl radical concentration can lead to elevation in p53 protein levels, p53 nuclear accumulation, p53-dependent cell growth arrest, and p53-dependent cell death in some cell types.

III. Studies on redox regulation of p53 *in vitro*

One of the possible mechanisms of p53 activation by ROI is direct oxidation of the p53 protein. Oxidation may activate p53 for cell cycle arrest and apoptosis. However, to date, all evidence from p53 oxidation studies conducted *in vitro* indicates that sequence specific DNA binding is inhibited by p53 oxidation (Hupp et al., 1992; Hainaut and Milner, 1993; Delphin et al., 1994; Sun and Oberley, 1996). Evidence that p53 cysteine residue oxidation can prevent p53 from properly binding its DNA consensus sequence comes from the fact that: (**i**) high concentrations of dithiothreitol (DTT) are required (0.1-10 mM) to allow recombinant p53 or p53 in nuclear extracts to bind DNA; (**ii**) treatment of purified recombinant p53 with the thiol alkylating agent N-ethyl maleimide (Rainwater et al., 1995) or *in vitro* translated p53 with diamine (Hainaut and Milner, 1993) prevents p53 from binding to its DNA consensus sequence. Thus, it appears that maintenance of p53 cysteine residues in the reduced state is necessary for optimal p53 consensus sequence-dependent DNA binding.

Another assay to test whether ROI may modulate p53 activity is the p53-transactivation assay. In this assay, a plasmid expressing p53 is cotransfected with a plasmid encoding a p53-responsive element placed upstream of a gene that codes for a transcription reporter. In one report it was demonstrated, using this assay, that H_2O_2 inhibited p53-mediated transactivation (Parks et al., 1997) consistent with the data demonstrating that oxidized p53 fails to bind DNA *in vitro*. This result, at the outset, appears inconsistent with data demonstrating that H_2O_2 treatment correlates with an increase in p53 protein and transactivation of the $p21^{WAF1/CIP1}$ gene (Chen et al., 1998). Indeed, nuclear extracts from cells treated with H_2O_2 contain higher levels of p53 sequence specific DNA binding activity than untreated cells (Verhaegh et al., 1997).

A possible explanation for this apparent contradiction is that H_2O_2 treatment of cells may directly oxidize p53 and, in addition, may lead to higher levels of p53 protein. The oxidized p53 is expected to be incapable of binding p53-dependent effector genes *in vivo*. However, it must be kept in mind that nuclear extracts derived from H_2O_2-treated cells often include DTT in the DNA-binding buffer. In this case, the high levels of oxidized p53 may be rapidly converted to a reduced form that can bind consensus sequence containing DNA. Rapid reduction of p53 by DTT may explain why the p53 DNA binding capacity appears higher in H_2O_2-treated cells. Notwithstanding this argument, one must still explain the apparent p53-dependent upregulation of $p21^{WAF1/CIP1}$ observed in cultured cells after H_2O_2 treatment. It is possible that H_2O_2 treatment immediately leads to high levels of transcriptionally inactive p53. After initial oxidation of p53, the oxidized cysteine residues on p53 may be reduced by

specific enzymes that are also activated by H_2O_2. Upon reduction, p53 may then upregulate $p21^{WAF1/CIP1}$. If this prediction is correct, one would expect that H_2O_2 treatment would lead to delayed activation of $p21^{WAF1/CIP1}$. Such a prediction is consistent with the fact that H_2O_2 treatment of cells leads to an increase in p53 protein at 1.5 h post-treatment and to $p21^{WAF1/CIP1}$ increase at 18 h post-treatment (Chen et al., 1998).

If this scenario is correct then the molecules required to maintain p53 in a reduced state may, in some instances, be limiting within the cell. This conjecture is supported, to some extent, by the fact that the DNA-binding activity of recombinant mouse p53 in freshly prepared nuclear extracts from baculovirus-infected insect cells is stimulated by treatment with DTT (Delphin et al., 1994). It is possible that p53 protein is overexpressed in insect cells relative to the reducing molecules needed to keep the p53 in the reduced state. This model raises the question of whether enzymes involved in reducing oxidized protein cysteine residues affect p53 activity.

IV. Enzymes that may be responsible for maintaining p53 in the reduced state

Redox control of p53 may be a chemical or enzymatic process. No consistent data has emerged to indicate the presence of a protein oxidizing enzyme in the cytoplasm. However, several enzymes appear to participate in reducing cytoplasmic protein disulfide linkages (Thomas et al., 1995; Rietsch and Beckwith, 1998). Thus, it is possible that redox regulation of p53 occurs by chemical oxidation and enzymatic reduction. Although no redox enzyme can be excluded from involvement, evidence to date indicates that there are two candidate enzymes responsible for maintaining p53 in a reduced state in eukaryotic cells: Ref-1 and thioredoxin reductase.

V. Ref-1

Redox factor-1, or Ref-1, was characterized as an activity from HeLa cell nuclear extracts that increased recombinant p53 binding to a p53 consensus sequence (Jayaraman et al., 1997). Ref-1 was previously shown to increase the activity of Fos-Jun heterodimer binding to DNA in a manner that depended on DTT (Xanthoudakis and Curran, 1992). Similar to p53, Fos and Jun are redox-sensitive transcription factors that directly bind DNA and upregulate transcription of genes involved in cell cycle progression (Abate et al., 1990). Ref-1 also possesses class II hydrolytic apurinic/apyrimidinic (A/P) endonuclease activity (Demple et al., 1991; Robson and Hickson, 1991). Because of this latter function, Ref-1 has also been assigned other names such as APE, APEX, and HAP-1. The redox regulation portion of Ref-1 and the endonuclease activity of Ref-1 lie within separate domains of the protein.

In the presence of DTT, Ref-1 stimulates consensus DNA binding of full-length p53 but this stimulatory activity is severely inhibited when a C-terminally truncated form of p53 (p53 lacking its 30 C-terminal amino acid residues) is used in the DNA-binding assay. This is somewhat unexpected because there are no cysteine residues within this C-terminal region of p53. However, the C-terminus does appear to normally negatively regulate the sequence specific DNA binding function of p53 (Hupp et al., 1992, 1993; Hupp and Lane, 1994). Ref-1 and p53 do not form a stable complex regardless of whether DNA is present. It is possible, then, that Ref-1 transiently associates within the terminal 30 amino acid residues of p53 and reduces oxidized p53 cysteine residues within the central-DNA binding domain of p53. Importantly, Ref-1 was observed to increase p53 transactivation activity in transient expression assays. When the Ref-1 endonuclease domain was removed, Ref-1's ability to stimulate p53 DNA binding activity was severely inhibited but not completely abolished. The data indicate that Ref-1 may stimulate p53 DNA binding activity through both, a non-redox and a redox mechanism. Genetic studies using *REF1* +/- and *REF1* +/+ mice in appropriate genetic backgrounds suggest that p53 activation in response to UV irradiation is dependent on Ref-1 (Meira et al., 1997).

VI. Thioredoxin reductase

Thioredoxin reductase is another enzyme that may be responsible for reducing p53 cysteine residues, either directly or indirectly. Thioredoxin reductase is a protein disulfide reductase that catalyzes NADPH-dependent reduction of the active site disulfide in oxidized thioredoxin, a small protein (12-14 kD), to a vicinal dithiol (Arner et al., 1999). The requirement of thioredoxin reductase for human p53 activity was identified in a genetic complementation study in the yeast strain *Schizosaccharomyces pombe* (Casso and Beach, 1996). In this yeast strain, ectopically expressed human p53 causes growth arrest (Bischoff et al., 1992). Casso and Beach (1996) found that a mutation in a yeast homologue of the human thioredoxin reductase gene (*trr1*) rescued p53-dependent growth arrest. p53-mediated transcription was also downregulated by this mutant allele of *trr1*. Mutant *trr1* required O_2 for its inhibitory effect on p53-mediated growth arrest. A strain of *S. pombe* lacking *trr1* acted in a similar manner to the strain expressing the mutant *trr1*, suggesting that the original mutant *trr1* acted as a dominant negative allele. The requirement for thioredoxin reductase in order to maintain the transcriptional activity of p53 was also demonstrated in the evolutionarily distant to *S. pombe* yeast strain *Saccharomyces cerevisiae* (Pearson and Merrill, 1998). These results suggested that either p53 itself, or a protein required for p53 function, is susceptible to disulfide bond formation. Once the disulfide bond is formed p53-mediated transactivation is abrogated. Thioredoxin reductase is required to reduce the disulfide bond and restore p53 function.

It is possible that some redox reactions are controlled by subcellular localization of redox-sensitive factors. Interestingly, translocation of cytoplasmic thioredoxin to the nuclear compartment of mammalian cells was recently demonstrated (Hirota et al., 1997). Furthermore, thioredoxin and Ref-1 can form a complex *in vitro* and *in vivo* . The

precise mechanism and the role of thioredoxin, Ref-1 and thioredoxin reductase in the regulation of p53 activity in mammalian cells is unknown but offers a potentially fertile environment for further experimental exploration.

VII. Potential sites of p53 cysteine residue oxidation-a structural analysis

In order for p53 cysteine residues to be redox regulated the sulfur atoms must be accessible to the oxidant. Structural studies can be used to rule out many potential cysteine oxidation sites based on solvent accessibility. Very few cysteine oxidation reactions on cytoplasmic proteins have been mapped, which might explain why no consensus sequence has emerged. To analyze the structural requirements for protein cysteine oxidation investigators have treated purified cytoplasmic proteins of known structures with glutathione disulfide, a compound that forms a mixed glutathione disulfide with protein cysteine residues (Thomas et al., 1995). In the case of rat liver carbonic anhydrase III, the crystal structure of the protein with conjugated glutathione was solved. For other proteins a two-part process was used to determine the structural requirements for glutathione conjugation. First, the glutathione-cysteine disulfide was mapped. Second, the mapped cysteine was assessed for solvent accessibility through analysis of the crystal structure of the unconjugated protein. In these studies it was determined that the residues surrounding the susceptible cysteine residues showed no consensus sequence. The only consistent feature found was that cysteine residues were located on the surface of the protein. Structure analysis of Fos and Jun bound to DNA also indicated that their oxidation-susceptible cysteine residues are exposed to the surface as well (Chen et al., 1998). The fact that solvent accessible cysteine residues are in close proximity to DNA binding residues may explain why Fos and Jun oxidation prevents them from binding to DNA.

We analyzed the crystal structure of residues 94-289 of p53 bound to DNA (Cho et al., 1994) in order to determine which of the 10 cysteine residues in this domain may be exposed to solvent (Connolly, 1983). As shown in **Fig. 2**, the sulfhydryl groups of Cys124, Cys176, Cys182, Cys229, Cys242 and Cys277 can theoretically react with small molecules on the surface of p53 (indicated with an asterisk). We conducted our analysis assuming that zinc was absent in this measurement. It is possible that part of the oxidation reaction involves removal of zinc as was recently shown for Hsp33 (Jakob et al., 1999).

To determine which p53 cysteine residues may form intramolecular disulfide linkages the distances between cysteine sulfur atoms were measured. Among the cysteine residues exposed to the solvent only Cys176 and Cys242 can theoretically form a disulfide bond (the interatomic distance of sulfur atoms is 3.66 Å). Once zinc is removed and Cys 176 and Cys242 become exposed and/or oxidized, either of them could attack Cys238, which also appears to be a reasonable candidate to participate in disulfide bond formation.

If p53 forms an intramolecular disulfide bond it is important to consider the relative orientation of the two cysteine residues in question. One measure of the degree of cysteine residue side chain movement required for disulfide bond formation is to compare the χ1 dihedral angles of p53 Cys176 and Cys242 to the χ1 dihedral angles of disulfides in known protein structures. In early work, Richardson (1981) measured the χ1 dihedral angles of 70 protein disulfide bonds and found that the χ1 angles tended to, but did not exclusively, cluster at -60° (±20°), +180 (±20°) and +60° (±20°). The relative frequency of these angles in protein disulfides was -60°>180°>+60°. In p53, the measured χ1 angle of Cys176 is +74° while that of Cys242 is -90°. Thus, the χ1 angle of Cys176 falls within the least frequent cluster of observed disulfide dihedral angles (+60° (±20°)) while the χ1 of Cys242 does not fall into any particular class. If the zinc atom is removed, the cysteine residue side chains may undergo slight changes to form more favorable dihedral angles for disulfide bond formation.

A third factor influencing the likelihood of disulfide bond formation is the polarity of the amino acid residues near the cysteine residue (Snyder et al., 1981). Residues that are positively charged and in close proximity to a cysteine residue could attract negatively charged disulfides, such as glutathione disulfide, to the reactive cysteine residue on p53. We note that many of the conserved cysteine residues on the surface of p53, Cys176, Cys242, Cys275 and Cys277 lie within a region of p53 that is electrostatically positively charged (**Fig. 3**). This is also the region of p53 that interacts with DNA. Based on our analysis of the crystal structure it is most plausible that Cys176 and Cys242 could form a disulfide bond. However, involvement of other cysteine residues in either intra- or inter-molecular disulfide bond formation can not be overlooked. In fact, it was demonstrated that recombinant p53 purified in the presence of chelex-treated solution (presumably metal ion-free) was unable to bind consensus sequence-containing DNA unless DTT was added (Rainwater et al., 1995). Apparently, zinc is tightly bound to p53 during this purification procedure. Interestingly, adding more zinc to the p53 did not enhance its DNA binding indicating that DTT may increase p53 binding to DNA by generating reduced free sulfhydryl groups on cysteine residues not responsible for zinc binding. In this regard, one group has reported that a small percentage of recombinant p53 could form a p53-p53 dimer that could be converted to a monomer after addition of DTT (Delphin et al., 1994).

If p53 redox control plays an important role in its regulation one would expect that the cysteine residues at the surface of the protein would be well conserved. An alignment of p53 amino acid sequences from 23 species shows that solvent accessible residues cysteine 176, 242 and 277 share nearly 100% identity (Soussi and May, 1996) but not other solvent accessible cysteine residues. Cysteine residues 242 and 277 are conserved throughout all 23 species reported while residue 176 is conserved in 22 species.

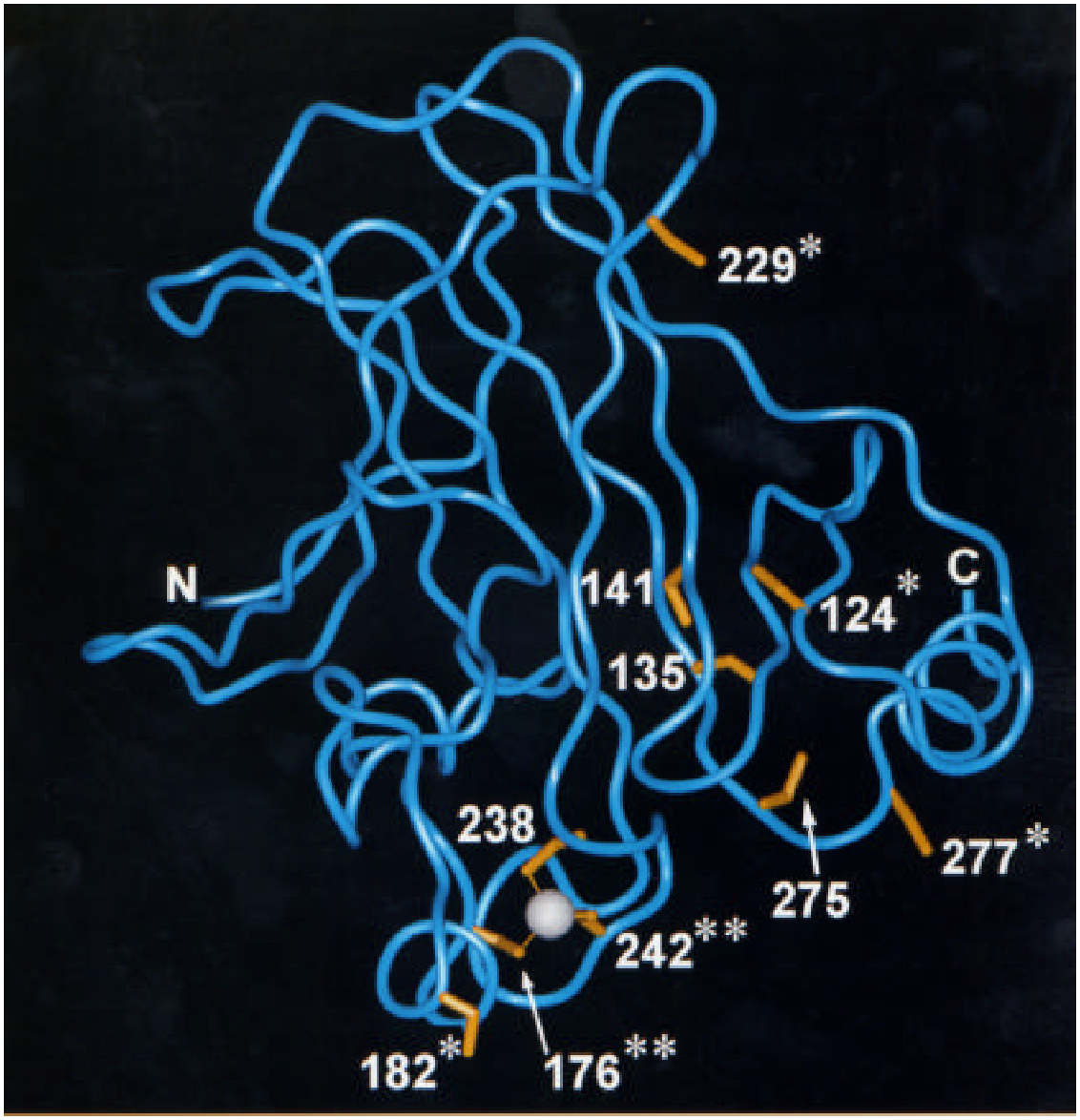

Figure 2. **Analysis of p53 (residues 94-289) for potential cysteine residue oxidation** (Cho et al., 1994). Backbone protein chain is in blue and cysteine side chains are in orange (only the bonds connecting the C_α and C_β and S atoms are represented). The white sphere represents a zinc atom. A thin line between the three cysteine S atoms and zinc are shown representing metal-sulfur bonds. Numbers refer to cysteine residues; the amino terminus and carboxyl terminus are represented by the 'N' and the 'C', respectively. The solvent exposed residues are Cys124 , Cys176, Cys182, Cys229, Cys242 and Cys277 (denoted by *). Cys176 and Cys242 can potentially form a disulfide bond (denoted by **). The structure was displayed and analyzed using the Insight II software, version 98.0 (Molecular Simulations Incorporated).

Only in one species (*Ovis aries*, sheep) does the p53 sequence reveal a change in Cys176 to Ser176 (Dequiedt et al., 1995). This is extremely interesting because a serine at this site is expected to prevent consensus DNA binding (Rainwater et al., 1995). Other conserved cysteine residues are maintained in sheep p53. **Fig. 4** shows a sequence alignment between human p53 and squid p53 (the most divergent of the p53 genes overall). We also show an alignment of the p53 cysteine coding region with two genes predicted to be similar to p53, p73 and p51/p63 (Kaghad et al., 1997; Osada et al., 1998; Yang et al., 1998). The nearby double positive charge on residues in close proximity to Cys176 may render this residue more susceptible to reaction with a negatively charged oxidizing molecule such as glutathione disulfide (Snyder et al., 1981). If one or more of these four cysteine residues is oxidized in p53 it is possible that oxidation may also alter the activity of p73 and p51/p63.

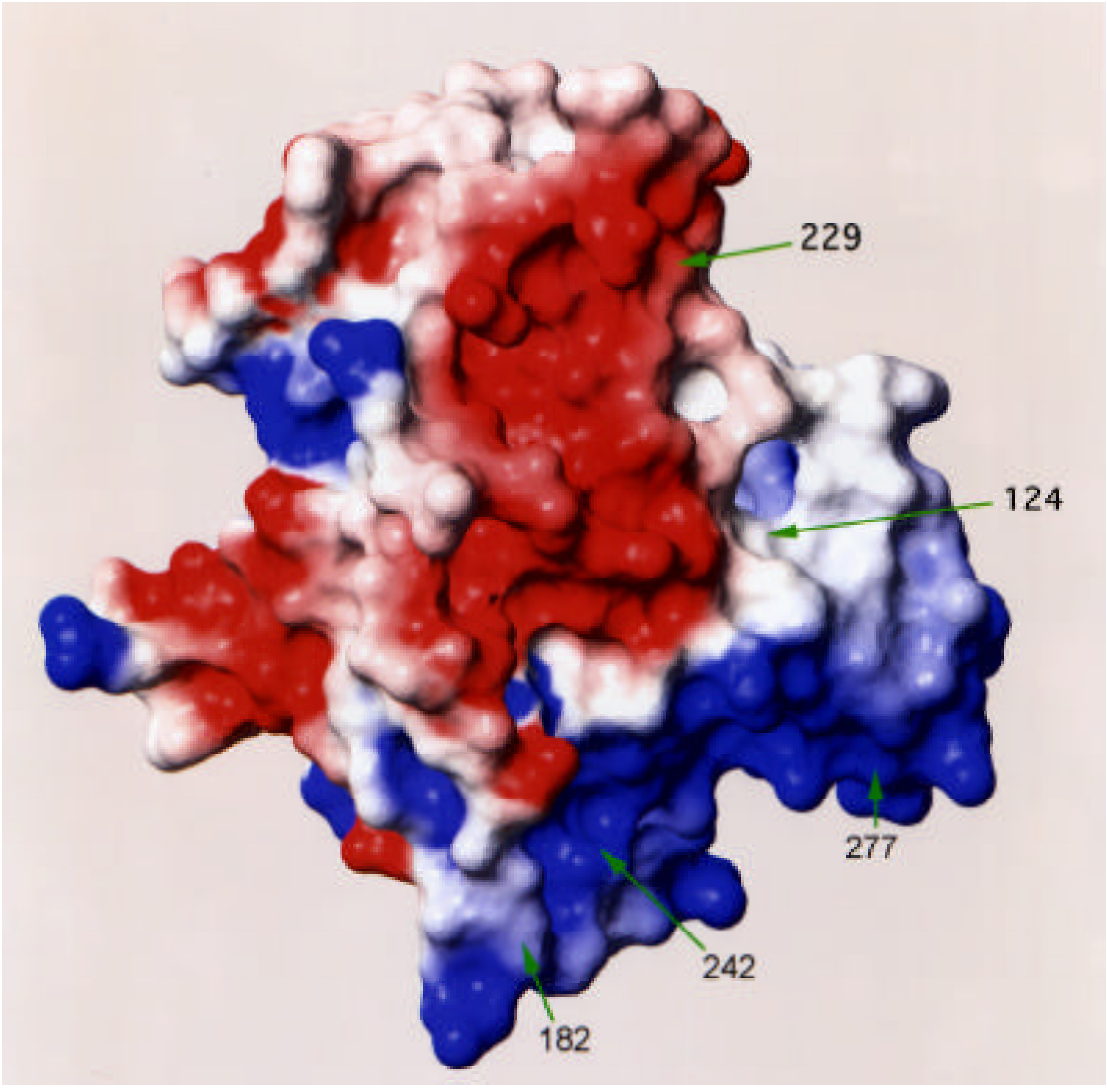

Figure 3. Surface electrostatic potential of p53. The crystal structure shown in Fig. 2 was analyzed for electrostatic potential using the MolMol program, version 2.6 (Koradi et al., 1996). Blue color represents atoms with low electron density, red color represents residues with high electron density and white represents neutral and charged residues. The arrows point to positions of the surface exposed sulfur atoms of the cysteine residues on the surface in this orientation. Note that the orientation of the p53 protein is identical to the p53 structure shown in **Fig. 2**. The sulfur atom of the Cys residue 176 was not exposed on this face of the protein.

p53 and p53-related proteins	Zn binding cys.	Zn binding cys.	Conserved domain V
human p53	171EVVRR**C**PHHER181	235NYMCNSS**C**MGG245	272VRVCA**C**PGR280
squid p53	197EVVKR**C**PNHEA206	261QFMCLGS**C**VGG271	297VRICA**C**PGR305
human p51/p63	200EVVKR**C**PNHEL210	266NFMCNSS**C**VGG276	303ARICA**C**PGR311
human p73	189DVVKR**C**PNHEL199	255NFMCNSS**C**VGG265	292GRICA**C**PGR301
conserved charged residues	- ++ -		+ +

Figure 4. Sequence comparison of well-conserved solvent exposed p53 cysteine residues. Cys residues expected to be solvent accessible are in boldface. Conserved charged residues within close proximity to the cysteine residues are also shown at the appropriate polarity and location.

Our structure analysis is based on the crystal structure of the p53 DNA binding domain bound to a p53-consensus sequence within an oligonucleotide (the B monomer, Cho et al., 1994). Although this structure is helpful in providing predictions of possible oxidation sites it may not provide the true picture of the conformations p53 exhibits in the cell. Several reports have shown that p53 is conformationally flexible and, depending on temperature and oligomeric status, it can also exist in different conformations *in vitro* (Ponchel and Milner, 1998; McLure and Lee, 1999). Within the crystal structure of p53 bound to DNA there are actually three monomers of p53 in the repeating subunit (named A, B and C). When the images of the monomers were superimposed differences in some of the cysteine residue side chain orientations were observed (data not shown). Thus, it is possible that the conformational changes of p53 observed in the cytosol could expose different cysteine sulfhydryl groups at the protein surface.

VIII. Mutational analysis of p53 cysteine residues

Based on the fact that oxidizing agents prevent p53 binding to consensus-sequence containing DNA and that some cysteine residues are well-conserved it is predicted that site-directed mutagenesis of these cysteine residues would alter p53 activities. Mann and coworkers have conducted a mutational study examining the importance of p53 cysteine residues in sequence-specific DNA binding, suppression of cell transformation and p53-mediated transactivation (Rainwater et al., 1995). A Cys to Ser substitution at each cysteine residue of murine p53 was created and the biochemical and biological activities of each individual cysteine mutant was compared to wild-type p53. For the sake of convenience, we will adopt the convention of enumerating the cysteine residues based on the human sequence.

From the relative activities in DNA binding, transactivation and transformation suppression, p53 cysteine residues were categorized into three groups. One group of cysteines (those at sites 176, 238 and 242) directly interact with zinc and are essential for DNA binding, transactivation and transformation activity of p53. A second group of cysteine residues (those at sites 124, 135, 141 and 275) is required for transactivation and suppression function. DNA binding activity of p53 is maintained when cysteine residues 124, 135, 141 and 275 are changed to serine. The third group (cysteine residues at sites 182 and 277) did not exhibit any alterations of the measured activities of p53 when the residues were changed to serine.

A cysteine to serine substitution is a very conservative change. An oxidation reaction resulting in a disulfide linkage may have a more dramatic consequences than a serine residue substitution. In this regard, it was shown that Cys to Ser substitution at a redox-sensitive Cys residue in c-Fos resulted in DTT-independent DNA binding (Okuno et al., 1993). Thus, oxidation at any of the three classes of cysteine residues may alter p53 activities.

IX. Metal binding agents may alter p53 redox level

A complicating factor in the field of redox chemistry is the fact that metal ions can catalyze the production of hydroxyl radicals. Thus, when studying redox changes, free transition metals (elements in groups IB, IIB, VIB, VIIB and VII in the periodic table) must always be taken into consideration. The biological systems have evolved molecules to bind free metals but the mechanisms by which metals are released by these systems are not clear. Metal ions, in combination with hydrogen peroxide, can form hydroxyl radicals through the Fenton reaction, also known as the metal-catalyzed Haber-Weiss reaction:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^{\bullet} + OH^{-} \quad (1)$$

$$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2 \quad (2)$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow OH^{\bullet} + OH^{-} + O_2 \quad (3)$$

The hydroxyl radical is an extremely reactive species. It reacts with most substances with diffusion-limiting rate constants (10^9- 10^{10} M^{-1} s^{-1}). Such reactivity implies a very short half-life and the molecule will likely be unable to travel at great distances. Hainaut has investigated the possibility that copper can modulate p53 DNA binding properties (Hainaut and Milner, 1993; Hainaut et al., 1995). Recombinant p53 was translated in rabbit reticulocyte lysate and exposed to Cu(II) sulfate at 30 µM. Copper exposure induced wild-type p53 to adopt a denatured conformation as detected by conformation-dependent antibodies. When Cu(II) was added to purified recombinant p53 there was no change in the electron spin resonance (ESR) spectrum indicating that Cu(II) may not bind to p53. However, when H_2O_2 was added to the Cu(II)/p53 mixture, a novel ESR signal, assigned to Cu(II), was observed indicating binding of Cu(II) to p53 under these conditions. Hainaut interpreted these data as follows: the added Cu(II) is initially reduced to Cu(I) by p53 cysteine residues thereby remaining ESR silent. After reduction, the Cu(I) is oxidized by H_2O_2 to Cu(II) but remains bound to p53. In support of Hainaut's model, the Cu(I) chelating agent, bathocuproinedisulfonic acid (BCS) protected the p53-DNA binding activity, an activity usually inhibited by Cu(II). DMSO, a hydroxyl radical scavenger, and sodium azide, a singlet oxygen scavenger, failed to prevent Cu(II)'s ability to inhibit p53's DNA binding activity ruling out the possibility that these two oxygen species are potential mediators of p53 oxidation.

Can copper mediate p53 oxidation in cultured cells? To test the idea that copper may mediate changes in p53 protein properties *in vivo* the cell permeable copper chelating agent, pyrrolidine dithiocarbamate (PDTC), was employed (Nobel et al., 1995; Verhaegh et al., 1997). This agent is similar in structure to dithiocarbamate-based herbicides, insecticides and fungicides commonly used in the pesticide industry (WHO, 1988). Treatment of cells with PDTC led to inhibition of p53 DNA binding activity and inhibition of stressor-mediated activation of p21 (presumably via p53

activation). Interestingly, PDTC-treated nuclear extracts were incapable of binding to the p53 consensus sequence DNA. The investigators went on to demonstrate that intracellular copper levels significantly increased in the presence of PDTC (Verhaegh et al., 1997). Wu and Momand (1998) demonstrated that PDTC treatment of cultured cells led to increased oxidation of p53 protein. Oxidation of p53 correlated with inhibition of p53-mediated transactivation, inhibition of p53 nuclear accumulation and inhibition of UV-induced p53 protein level elevation in fibroblasts. Interestingly, PDTC treatment also prevented E6-mediated degradation of p53. These results suggested that PDTC-mediated copper loading into cells may directly oxidize p53 or generate hydroxyl radicals near the surface of p53, resulting in cysteine residue oxidation.

We have incorporated the experimental data into a coherent testable mechanism for p53 oxidation. Because a high level of reduced glutathione is present in the cytosol we have included glutathione as a transporter of electron radicals. This reaction mechanism is based on a combination of previous studies conducted, primarily, on oxidation of cysteine (Gerweck et al., 1984; Winterbourn, 1993; Thomas et al., 1995):

PDTC-Cu^{2+} transport across cell membrane (**1**)

$p53\text{-}SH + Cu^{2+} \rightarrow p53\text{-}S^{\cdot} + Cu^{1+} + H^{+}$ (**2**)

$p53\text{-}S^{\cdot} + GSH \rightarrow (p53\text{-}S\text{-}S\text{-}G)^{\cdot -} + H^{+}$ (**3**)

$(p53\text{-}S\text{-}S\text{-}G)^{\cdot -} + O_2 \rightarrow p53\text{-}S\text{-}S\text{-}G + O_2^{\cdot -}$ (**4**)

$O_2^{\cdot -} + 1/2H_2O \rightarrow 1/2O_2 + H_2O_2$ (catalyzed by SOD) (**5**)

$H_2O_2 \rightarrow H_2O + 1/2O_2$ (catalyzed by catalase) (**6**)

$p53\text{-}SH + Cu^{2+} + GSH \rightarrow p53\text{-}S\text{-}S\text{-}G + Cu^{1+} + 2H^{+}$

According to the reaction mechanism, PDTC transports Cu^{2+} into the cell (**Reaction 1**). The p53 cysteine residues are directly oxidized by p53 bound cupric ion (**Reaction 2**). After one-electron oxidation of p53 the p53 cysteine residue carries a thiyl radical that rapidly reacts with free glutathione (GSH) to form the disulfide radical (**Reaction 3**). This radical is very unstable and requires oxidation before it reforms the reactants. Molecular oxygen oxidizes the radical to form the S-glutathiolated form of the p53 cysteine residue (**Reaction 4**). The superoxide formed by one-electron reduction of oxygen reacts with water to form hydrogen peroxide by superoxide dismutase (**Reaction 5**). Hydrogen peroxide is then dissociated into water and molecular oxygen by catalase (**Reaction 6**). In the future, it will be important to test this reaction mechanism *in vitro.*

While free metal ions may lead to p53 oxidation it is also possible that PDTC indirectly leads to p53 oxidation by chelating the zinc atom that is bound to Cys176, Cys238 and Cys242. After zinc removal the p53 cysteine residues may be oxidized by another molecule, possibly hydroxyl radical. Both, *in vitro* and *in vivo* evidence support the idea that zinc is required for p53-mediated consensus-sequence binding (Pavletich et al., 1993; Rainwater et al., 1995; Verhaegh et al., 1998) Evidence in support of chelation of zinc leading to p53 conformational changes in cultured cells has recently been demonstrated with a membrane permeable zinc-specific chelator N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) (Verhaegh et al., 1998). Nuclear extracts from TPEN-treated cells do not bind consensus DNA. However, if zinc is supplemented into the TPEN-treated media prior to cell harvesting p53 retains the ability to bind DNA. TPEN alters the conformation of p53 protein. Importantly, TPEN does not affect the DNA binding activity of Oct-1, another transcription factor that does not require zinc. The data suggests that metal ions can modulate p53 transactivation activity, prevent consensus DNA binding by p53, and can alter the p53 protein conformation. Whether zinc chelation by TPEN leads to p53 oxidation is unclear at the moment.

X. Other functions of p53 that may be regulated by redox levels

Several studies indicate that p53 oxidation prevents its ability to bind consensus sequence containing DNA. This leads to the possibility that oxidation may be a mechanism of p53 down-regulation during oxidative stress. Oxidation of p53 may constitute a mechanism to turn off p53-mediated transactivation after its checkpoint function has been fulfilled and DNA has been repaired. It is also possible that oxidation regulates p53 function in a positive aspect. Aside from its role as a transcription factor, biochemical and genetic studies have demonstrated that p53 is responsible for the faithful execution of other activities directly related to maintenance of genome stability. Such activities include global genomic nucleotide excision repair (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997; Ford et al., 1998) and inhibition of homologous recombination (Mekeel et al., 1997; Dudenhoffer et al., 1998). The p53 activities related to repair and recombination may result, in part, from direct interaction of p53 protein with DNA. Some DNA binding studies suggest that p53 has a direct role in DNA repair. For example, p53 binds to insertion/deletion mismatches (Lee et al., 1995; Szak and Pietenpol, 1999), single stranded DNA molecules, and can mediate DNA strand exchange reactions (Bakalkin et al., 1994, 1995; Reed et al., 1995; Wu et al., 1995).

The idea that oxidized p53 may bind non-consensus DNA and mediate a function has not been extensively explored. Insertion/deletion mismatch DNA mutations may result from polymerase-induced errors during replication. Mann and coworkers have shown that recombinant p53 can bind to insertion/deletion mismatch DNA in the presence or absence of DTT (Parks et al., 1997). This may indicate that this particular DNA binding function of p53 may be preserved under oxidizing conditions. Furthermore, p53 has recently been demonstrated to possess double-strand DNA exonuclease activity (Mummenbrauer et al., 1996; Janus et al., 1999), which may also be related to a repair function. The p53 protein has been shown to bind substrates that

mimic recombination intermediates including three stranded DNA molecules (Dudenhoffer et al., 1998) and Holliday junctions (Lee et al., 1997). Some p53 mutations found in human tumors produce mutant p53 proteins that disrupt DNA repair and recombination activities. Whether p53 oxidation modulates these different p53 activities is unclear at the moment.

XI. Conclusion

Redox regulation of p53 activity was proposed in 1993 based on the observation that sequence specific DNA binding could be inhibited by agents that blocked sulfhydryl groups (Hainaut and Milner, 1993). However, p53 redox regulation is still poorly understood. The fact that p53 activation in cultured cells can be promoted by agents that induce the formation of ROI while the oxidation of p53 protein inhibits its ability to bind consensus sequence-containing DNA *in vitro* renders this regulation mechanism even more intriguing. Two disulfide-reducing proteins, Ref-1 and thioredoxin reductase may hold the key to understanding this regulation. By analyzing the structure of p53, we found several well-conserved cysteine residues exposed at the protein surface. Another area of investigation into p53 redox control is related to its ability to bind metal ions. Binding of metal ions may directly affect p53 redox potential either at the zinc binding cysteine residues or at other cysteine residues on the protein surface. To date, most evidence suggests that p53 oxidation inhibits p53 activity. However, *in vitro* studies show that oxidized p53 retains the ability to bind insertion/deletion mismatches in DNA without the addition of DTT. This opens the possibility that redox regulation can be used as a molecular switch inside the cell to promote other functions of p53 that are less well-known (Parks et al., 1997). Future studies on the prevalence of p53 oxidation in response to stressors and the effects of p53 protein oxidation on its biochemical activities are clearly needed to address these issues.

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