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Regulation of p53 by Metal Ions and by Antioxidants: Dithiocarbamate Down-Regulates p53 DNA-Binding Activity by Increasing the Intracellular Level of Copper

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Mutations in the p53 tumor suppressor gene frequently fall within the specific DNA-binding domain and prevent the molecule from transactivating normal targets. DNA-binding activity is regulated in vitro by metal ions and by redox conditions, but whether these factors also regulate p53 in vivo is unclear. To address this question, we have analyzed the effect of pyrrolidine dithiocarbamate (PDTC) on p53 DNA-binding activity in cell lines expressing wild-type p53. PDTC is commonly regarded as an antioxidant, but it can also bind and transport external copper ions into cells and thus exert either pro- or antioxidant effects in different situations. We report that PDTC, but not *N*-acetyl-L-cysteine, down-regulated the specific DNA-binding activity of p53. Loss of DNA binding correlated with disruption of the immunologically "wild-type" p53 conformation. Using different chelators to interfere with copper transport by PDTC, we found that bathocuproinedisulfonic acid (BCS), a non-cell-permeable chelator of Cu^{1+}, prevented both copper import and p53 down-regulation. In contrast, 1,10-orthophenanthroline, a cell-permeable chelator of Cu^{2+}, promoted the redox activity of copper and up-regulated p53 DNA-binding activity through a DNA damage-dependent pathway. We have previously reported that p53 protein binds copper in vitro in the form of Cu^{1+} (P. Hainaut, N. Rolley, M. Davies, and J. Milner, Oncogene 10:27–32, 1995). The data reported here indicate that intracellular levels and redox activity of copper are critical for p53 protein conformation and DNA-binding activity and suggest that copper ions may participate in the physiological control of p53 function.

The tumor suppressor protein p53 is a transcription factor regulating cell cycle progression, cell survival, and DNA repair in cells exposed to genotoxic stress. Exposure to DNA damage induces p53 to accumulate in the nucleus, to bind to specific DNA sequences, and to transactivate several sets of target genes including effectors of the cell cycle and apoptosis such *p21/WAF1/Cip1* (10, 11), *bax* (31), *mdm-2* (24), *gadd45* (25), and *cyclin G* (37). However, the exact mechanisms by which p53 "senses" DNA damage are still unknown. In many cell types, activation of p53 leads to transient cell cycle arrest in G_1 or at the G_2/M border. In other cells, accumulation of p53 triggers apoptosis. Both of these responses suppress the proliferation of cells that have undergone DNA damage and contribute to prevent the propagation of cells with potentially oncogenic mutations (13).

In human cancers, the capacity of p53 to transactivate gene expression is frequently impaired by missense mutation in the DNA-binding domain. This domain (residues 102 to 292) is made of two β-sheets supporting two loops and a loop-sheet-helix motif, which form the DNA-binding surface (7). The two loops, L2 and L3, are connected by a zinc atom tetrahedrally coordinated on residues Cys176, His179, Cys238, and Cys242. The DNA-binding domain shows a remarkable flexibility, underlined by the intrinsic temperature sensitivity for conformation of the p53 polypeptide (17, 18). This conformational flexibility can be analyzed with monoclonal antibodies PAb1620 (plus PAb246 for mouse p53) and PAb240. The PAb1620 epitope is exposed on the form of wild-type p53 that is competent for DNA binding and depends on the tertiary folding of the protein. In contrast, the PAb240 epitope (residues 213 to 217) (43) is cryptic in the form of the protein that is competent for DNA binding but is exposed by denaturation (17, 19). Many p53 mutants have lost reactivity with PAb1620 and react with PAb240, an observation which has led to the definition of two alternative p53 conformations, wild type ($PAb1620^+$ $PAb240^-$) and mutant ($PAb1620^-$ $PAb240^+$) (30).

In vitro, p53 DNA-binding activity is allosterically controlled by interaction with DnaK (the *Escherichia coli* homolog of Hsp70), by phosphorylation, and by reduction of cysteines (15, 17, 20–23). Moreover, in vitro chelation of zinc abrogates p53 DNA-binding activity, induces the wild-type protein to adopt a $PAb1620^-$ $PAb240^+$ form, and promotes oxidation of cysteines, suggesting that p53 conformation is affected by metalloregulation of zinc and by redox regulation (16–18, 22). Similar conformational effects have been observed in the presence of the cell-permeable zinc chelator TPEN (*N*,*N*′-tetrakispyrimidylethylenediamine) in intact cells, indicating that p53 activity in cultured cells is also dependent on the availability of zinc (46).

Several transcription factors, such as NF-κB and AP-1, are redox regulated in vivo (42). For example, low levels of reactive oxygen species act as "second messengers" to activate NF-κB, and this activation can be blocked by several antioxidants, such *N*-acetyl-L-cysteine (NAC), pyrrolidine dithiocarbarmate (PDTC), and thioredoxin (41). In addition, DNA binding by NF-κB in vitro requires the reduction of cysteinyl residues (44) and is activated in vivo by stimuli which affect the intra-

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cellular redox status, such as oxidants, UV and γ radiation, hypoxic stress, and inflammatory cytokines (40). The transcription factors c-*fos* and c-*jun* are also redox regulated in vivo. While the transcription of c-*fos* and c-*jun* is activated by both oxidants and antioxidants, the DNA-binding activity of AP-1 is activated by the reduction of a single cysteine in c-Fos and c-Jun proteins by the redox enzyme Ref-1 (48).

In this study, we have investigated whether p53 could also be controlled by redox regulation in intact cells by analyzing the effects on p53 of the dithiocarbamate PDTC, a metal-chelating compound that can exert either antioxidant or prooxidant effects in different situations (35). For example, PDTC inhibits the activation of NF-κB as well as the initiation of apoptosis in HL60 cells exposed to DNA-damaging agents, a property attributable to the capacity of PDTC to scavenge reactive oxygen species (4, 45). On the other hand, PDTC has also been shown to promote oxidation of glutathione and apoptosis in thymocytes, an activity attributed to the capacity of PDTC to exert prooxidant effects by increasing the intracellular level of redox-active copper (35). Here we show that PDTC altered p53 protein conformation and down-regulated p53 DNA binding in several cell lines expressing wild-type p53, including the breast cancer cell line MCF-7 and two human lymphoblastoid cell lines immortalized with EBV, IARC1104 (derived from a healthy donor) and GM3189 (derived from an ataxia-telangiectasia patient). Using metal chelators to interfere with copper transport, we show that the effects of PDTC on p53 were correlated with intracellular accumulation of copper but did not result from a drastic alteration of the intracellular redox status. Addition of PDTC together with hydrogen peroxide, a typical inducer of DNA strand breaks that activates p53, did not prevent DNA damage but significantly delayed the DNA damage-dependent induction of p53. This study shows that copper ions are capable of modulating p53 function in intact cells and provides evidence that p53 protein function is dependent upon metalloregulation and oxidation-reduction in vivo.

MATERIALS AND METHODS

Cell lines and cell culture. The human breast carcinoma cell line MCF-7 was cultured at 37°C under 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (PAA, Linz, Austria), 2 mM L-glutamine, and antibiotics. The Epstein-Barr virus-transformed lymphoblastoid AT homozygote cell line GM3189 was obtained from the National Institute of General Medical Science Mutant Cell Repository (Camden, N.J.). The Epstein-Barr virus-transformed normal lymphoblastoid cell line IARC1104 was provided by G. Lenoir, International Agency for Research on Cancer. Lymphoblastoid cell lines were cultured at 37°C under 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics.

Cell treatments. MCF-7 cells were drug treated at 70 to 80% confluency. Lymphoblastoid cell lines were treated as described by Artuso et al. (1). The cells were treated for 2 h with bathocuproinedisulfonic acid (BCS), $CuSO_4$, H_2O_2, *N*-acetyl-L-cysteine (NAC), 1,10-orthophenanthroline (OP), PDTC, $ZnCl_2$, or combinations thereof. All chemicals were purchased from Sigma, unless otherwise stated. PDTC and NAC were dissolved in phosphate-buffered saline (PBS) at 1 M and flushed with argon to prevent oxidation.

Nuclear protein extraction. Control and treated cells were washed twice with ice-cold PBS and collected by scraping (MCF-7) or centrifugation (lymphoblastoid cells). Nuclear extracts were prepared as described by Funk et al. (12) with the following modifications: the cells were lysed for 15 min on ice in extraction buffer A (100 μl/10^6 cells) (20 mM HEPES [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% Nonidet P-40) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml 2 μg of aprotinin per ml, 0.7 μg of pepstatin A per ml). The nuclei were collected by centrifugation at 300 × *g* for 4 min, lysed for 30 min on ice in extraction buffer B (50 μl/10^6 cells) (same as buffer A but with 0.5 M instead of 10 mM NaCl), and then centrifuged for 15 min at 15,000 × *g* and 4°C. The supernatant was designated the nuclear fraction and kept at −80°C.

Detection of p53 and p21^{WAF1} proteins by Western blot analysis. Nuclear extracts (30 μg) were resolved on sodium dodecyl sulfate–10% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed with an enhanced chemiluminescence (Amersham) detection system in accordance with the instructions of the manufacturer. For p53 detection, the first antibody was DO-7 (Dako) at 250 ng/ml; for p21^{WAF1} detection, the WAF1 antibody (Oncogene Science) was used at 500 ng/ml. Peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce) at 250 ng/ml was used as the second antibody.

Electrophoretic gel mobility shift assays (EMSAs). The double-stranded p53 consensus binding sequence p53^{con} (5′-GGACATGCCCGGGCATGTCC-3′) (12) and an oligonucleotide containing the Oct-1 binding sequence (underlined; (5′-GACCACCTGGGTAATTTGCATTTCTAAAATA-3′) (33) were end labelled with ~3,000 Ci of [γ-^{32}P]ATP (Amersham) per ml.

The binding assay mixtures contained ^{32}P-labelled oligonucleotide (0.5 ng), nuclear extract (10 μg), sonicated salmon sperm DNA (2 μg; Promega), bovine serum albumin (5 μg), DTT (4 mM), and PAb421 (100 ng) and were adjusted to a final volume of 30 μl with buffer A. The mixtures were incubated for 30 min at 20°C. The specificity of the binding reaction was demonstrated by competition experiments with an excess of unlabelled p53^{con}. A 15-μl aliquot of each reaction mixture was loaded onto a 4% nondenaturing polyacrylamide gel and run in Tris-borate-EDTA (TBE) at 120 V for 2 to 3 h. The gels were then fixed, dried, and exposed to KODAK X-ray films at −80°C for 12 to 48 h.

Conformation-specific immunoprecipitation of p53. Control and treated cells were washed with ice-cold PBS, lysed in immunoprecipitation buffer (10 mM Tris [pH 7.6], 140 mM NaCl, 0.5% NP-40, protease inhibitors as above) for 20 min on ice, and cleared from cell debris by centrifugation. To remove nonspecific binding, the supernatant was cleared with 10% (wt/vol) *Staphylococcus aureus* protein A suspension (50 μl) for 20 min on ice, followed by centrifugation. For immunoprecipitation of p53, 1 μg of the monoclonal antibodies PAb1620 (wild-type specific), PAb240 (mutant specific), or PAb1801 (recognizing both wild-type and mutant p53) was added to the cleared supernatant for 2 h at 4°C. Immune complexes were collected with *S. aureus*-protein A suspension and washed five times with immunoprecipitation buffer. Immunoprecipitated p53 was recovered by resuspending the pellets in Laemmli buffer, and the protein was detected by Western blotting with the rabbit antibody CM-1 (Novocastra) as first antibody (1:1,000 dilution) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce) as the second antibody (250 ng/ml).

Analysis of intracellular redox status and of DNA strand breaks. The activities of antioxidant enzymes (catalase and manganese-dependent and copper/zinc-dependent superoxide dismutases) and the levels of reduced versus oxidized glutathione were measured as described previously (38). For the determination of zinc, copper, and iron, cells were trypsinized, washed three times with 400 mM isotonic Tris (pH 7.3, treated with chelex resin to minimize contamination by exogenous metals), and resuspended in hypotonic Tris (10 mM). The cell suspensions then underwent five cycles of freezing (liquid nitrogen) and thawing (37°C). After removal of aliquots for protein determination, copper, iron, and zinc concentrations were determined by electrothermal atomic absorption spectrophotometry with a Perkin-Elmer 3030 spectrophotometer for determination of zinc and a Hitachi 28270 spectrophotometer for determination of iron and copper. Strand breaks were determined by the alkaline unwinding technique as described previously (38).

Cell cycle analysis by flow cytometry. Cell nuclei were collected and stained with propidium iodide, using the cycleTEST-PLUS DNA-staining kit, as specified by the manufacturer (Becton Dickinson). The DNA contents of the stained nuclei were measured on a FACScan flow cytometer, and the results were analyzed with CellFIT software (Becton Dickinson).

RESULTS

PDTC down-regulates p53 DNA-binding activity in MCF-7 cells. Many oxidants induce intranuclear accumulation of p53, probably as a result of oxidative DNA damage. To examine whether antioxidants could modulate p53 induction, we used the cell line MCF-7, a breast cancer cell line which contains wild-type p53 alleles and expresses high levels of p53 protein in the PAb1620$^+$ (wild-type) conformation (8). Although these cells cannot be considered to have normal p53 function, there is evidence that the p53-dependent pathways are at least partially functional in MCF-7 cells (14). MCF-7 cells were exposed for 2 h to the oxidant H_2O_2 (200 μM), with or without 10 to 90 mM NAC (a precursor of glutathione) and 20 to 60 μM PDTC. None of these treatments affected the viability of MCF-7 cells, although longer exposure to PDTC (more than 12 h) had significant cytotoxic effects (data not shown).

Western blot analysis showed that the addition of NAC did not affect p53 levels in unexposed cells or prevent p53 accumulation in cells exposed to H_2O_2. In contrast, PDTC prevented the accumulation of p53 induced by H_2O_2 (Fig. 1A). By EMSA, we found that PDTC, but not NAC, decreased the basal DNA-binding activity of p53 and down-regulated p53

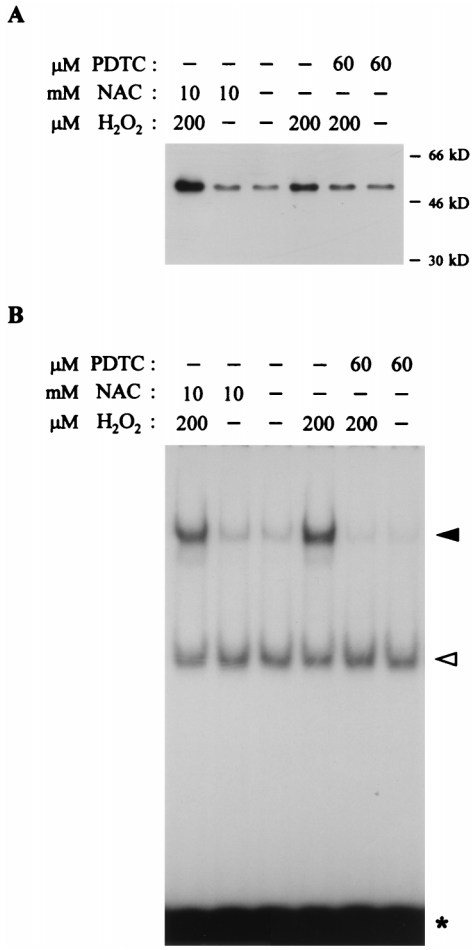

FIG. 1. Effects of PDTC and NAC on the H_2O_2-induced activation of p53. (A) Effect of PDTC and NAC on the H_2O_2-induced p53 protein accumulation. MCF-7 cells were treated with 200 μM H_2O_2, 10 mM NAC, 60 μM PDTC, and the indicated combinations thereof for 2 h prior to harvesting. The amounts of p53 protein were determined by Western blot analysis. (B) Effect of PDTC and NAC on the H_2O_2-induced DNA-binding activity of p53. Nuclear extracts of MCF-7 cells treated as described above were incubated with a ^{32}P-labelled double-stranded oligonucleotide, corresponding to the p53 consensus binding-sequence ($p53^{CS}$), and analyzed by EMSA. All reactions were carried out in the presence of PAb421, which stabilizes and supershifts p53-DNA complexes. The black arrowhead indicates the p53-DNA-PAb421 complexes, and the white arrowhead indicates the position of complexes that do not contain p53. This complex corresponds to an unidentified protein that binds to DNA in a non-sequence-specific manner. The asterisk marks the free probe.

DNA binding after exposure to H_2O_2 (Fig. 1B). Down-regulation of p53 by PDTC was also observed in other cell lines expressing wild-type p53, including the lung adenocarcinoma cell line A549 (data not shown) and the lymphoblastoid cell lines IARC1104 and GM3189 (see Fig. 5). Comparative Western blot analysis of nuclear and cytoplasmic extracts did not reveal any change in the intracellular distribution of p53 after exposure to PDTC (data not shown). These data thus indicate that in the absence of H_2O_2, PDTC does not affect p53 protein levels but impairs its capacity to bind to DNA.

PDTC alters the conformation of wild-type p53. The capacity of p53 to bind to specific DNA correlates with the adoption of the "wild-type" conformation, being reactive with the monoclonal antibody PAb1620 and nonreactive with PAb240. In vitro, agents that destabilize this conformation also impair p53 DNA binding (17). To determine the effect of PDTC on the p53 conformation, the protein was immunoprecipitated with PAb1620 and PAb240 from extracts of MCF-7 cells exposed to 60 μM PDTC, and the precipitates were analyzed by Western blotting with the rabbit polyclonal antibody CM-1 (see Materials and Methods). Figure 2 shows that PDTC altered the conformation of p53, as shown by decreased reactivity with PAb1620 and increased reactivity with PAb240. This conformational change was only partial, since a significant proportion of the protein retained PAb1620 reactivity and was not maximized by using higher concentrations of PDTC (data not shown). These data indicate that PDTC destabilizes the conformation of wild-type p53, an effect which is compatible with the decreased DNA-binding capacity observed in Fig. 1B. We and others have shown that the in vitro incubation of p53 with thiol oxidants or antioxidants induces conformational changes, correlated with changes in DNA-binding activity. For example, the antioxidant DTT increased the reactivity of p53 with PAb1620 and stimulated DNA binding, whereas the oxidant diamide converted p53 to the $PAb1620^{-}$ $PAb240^{+}$ form and inhibited DNA binding (17, 22). To determine whether PDTC could exert a redox effect on p53, wild-type p53 was translated in vitro and exposed to PDTC (5 to 100 μM). In contrast to DTT and diamide, PDTC did not affect the conformation or the DNA-binding activity of p53 translated in vitro (data not shown). This suggests that in intact cells, PDTC does not exert direct effects on the p53 redox status but acts indirectly by affecting a cellular pathway that regulates p53 activity.

PDTC mediates the accumulation of copper ions into MCF-7 cells. To analyze the effects of PDTC on the intracellular redox status of MCF-7 cells, we determined the levels of reduced versus oxidized glutathione, of membrane lipid peroxidation, and of endogenous metal ions such as zinc, iron, and copper. Only the level of intracellular copper was found to be significantly affected by PDTC (Table 1). Exposure to PDTC resulted in a 2.5-fold increase in the intracellular concentration of copper but not of zinc or iron. The increase in the copper concentration was blocked by addition to the culture medium of BCS, a specific Cu^{1+} chelator that does not cross the plasma membrane (26, 35). Exposure to BCS alone did not alter copper levels (Fig. 3). These results indicate that PDTC can chelate trace amounts of copper in the culture medium, transport copper across plasma membranes, and induce its intracellular accumulation. Addition of copper alone in the medium (in the form of $CuSO_4$ at concentrations of up to 300 μM) did not result in increased intracellular copper levels, whereas addition of both PDTC and micromolar amounts of $CuSO_4$ increased the intracellular copper levels by 2 orders of magnitude (Fig. 3) and induced acute cytotoxicity (data not shown). Our data are compatible with those of Nobel et al. (35), who demonstrated the capacity of PDTC to induce the accumulation of copper in thymocytes. However, in thymocytes, accumulation of copper was accompanied by rapid depletion of the glutathione pool

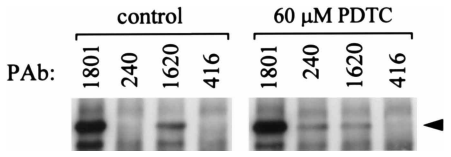

FIG. 2. Analysis of p53 protein conformation after exposure of MCF-7 cells to PDTC. MCF-7 cells were treated with 60 μM PDTC for 2 h. Cell lysates were immunoprecipitated with the monoclonal antibodies PAb1620 (wild-type specific), PAb240 (mutant specific) and PAb1801 (both forms of p53). The antibody PAb416 (large T antigen) was used as a negative control. Immunoprecipitated p53 was detected by Western blotting with the polyclonal p53 antibody CM-1. The black arrowhead indicates the position of p53.

TABLE 1. Levels of glutathione, metal ions, and lipid peroxidation in MCF-7 cells exposed to PDTC

Treatment	Glutathione concn (μmol/g of protein)[a]		Metal ion concn (μg/g of protein)[b]			MDA concn (μmol/g of protein)[b]
	Oxidized	Reduced	Zinc	Copper	Iron	
Control	3.92	173	245 ± 22	6.9 ± 0.9	104.9 ± 8.6	0.09 ± 0.01
PDTC (40 μM)	3.99	172	260 ± 15	18.7 ± 0.7	125.2 ± 13.8	0.10 ± 0.02

[a] Mean of two independent experiments.
[b] Mean and standard deviation of three independent experiments. MDA, malondialdehyde-thiobarbituric acid adducts (products of lipid peroxidation).

followed by apoptosis. The last two effects were not observed in MCF-7 cells. The absence of significant effects on the glutathione pool and on lipid peroxidation suggests that in contrast to thymocytes, PDTC does not have a powerful prooxidant effect in MCF-7 cells. This difference may be attributed to the constitutively high level of reduced glutathione in MCF-7 cells: these cells may be more resistant than thymocytes to the prooxidant effect of PDTC.

Down-regulation of p53 by PDTC correlates with copper import. We have previously shown that copper can bind to p53 in the form of Cu^{1+} and that low levels of copper (5 to 20 μM) could alter p53 protein conformation and DNA binding activity in vitro (18). To determine whether the effects of PDTC on p53 result from intracellular accumulation of copper, we have analyzed the DNA-binding activity in cells exposed to PDTC and BCS, which blocks PDTC-mediated copper transport. The results show that the addition of BCS prevented the down-regulation of p53 activity induced by PDTC whereas BCS alone has no effect (Fig. 4A). These data suggest that PDTC down-regulates p53 by increasing the intracellular level of copper. In contrast to p53, Oct-1, a ubiquitous transcription factor which is not under redox control in vivo, was not affected by PDTC. Thus, the effect of PDTC on p53 does not reflect a general perturbation of protein-DNA interactions (Fig. 4B).

Copper is a redox-active metal that exists in at least two major oxidation states, Cu^{1+} and Cu^{2+}, with distinct biochemical activities in vivo. Redox cycling of copper between Cu^{1+} and Cu^{2+} catalyzes the intracellular production of hydroxyl radicals ($^{\bullet}OH$), a property which explains the clastogenic activity of copper in vitro and in intact cells. Complex formation with specific chelators can either inhibit or promote redox cycling by copper. For example, BCS prevents $^{\bullet}OH$ formation by stabilizing Cu^{1+}, whereas 1,10-orthophenanthroline (OP) enhances the efficiency of copper-dependent $^{\bullet}OH$ formation. The $Cu^{2+}(OP)_2$ complex thus behaves as a "chemical nuclease" and causes DNA strand breaks in vitro and in isolated cell nuclei (6). To further analyze the role of copper accumulation in the control of p53, MCF-7 cells were incubated for 2 h with OP (200 μM). OP induced a twofold increase in the level of intracellular copper, and this effect was additive to that of PDTC (Fig. 3). However, in contrast to PDTC, OP stimulated the DNA-binding activity of p53, whereas addition of both OP and PDTC had an intermediate effect (Fig. 4A; see also Fig. 5). Thus, despite having similar effects on intracellular copper levels, PDTC and OP had opposite effects on the DNA-binding activity of p53. These data indicate that the effects of OP and PDTC cannot be attributed solely to their capacity to increase intracellular copper but also depend upon the redox activity of the metal-chelator complex.

Activation of p53 DNA binding by OP results from DNA damage and requires the ATM protein. Recent studies have shown that OP stimulates DNA fragmentation in isolated rat liver nuclei by promoting the redox activity of endogenous copper ions (6). In agreement with this observation, we found that exposure of MCF-7 cells to OP induced the formation of DNA strand breaks, as determined by the rate of alkaline unwinding of DNA (data not shown). Induction of p53 after formation of DNA strand breaks requires the function of the ATM protein, which is defective in patients with ataxia-telangiectasia (AT) (1, 25). To determine whether p53 induction by OP was a consequence of DNA strand break formation, we compared the effects of PDTC and OP on p53 DNA-binding activity in two lymphoblastoid cell lines derived from a normal individual or from an AT patient (Fig. 5). In both cell lines, PDTC down-regulated p53 activity, and this effect was prevented by the addition of BCS. In the normal cell line, OP up-regulated p53 activity to a level similar to that observed with H_2O_2. In contrast, in the AT cell line, induction of p53 by H_2O_2 was partially impaired and no induction was seen with OP. These data indicate that cells from AT patients are deficient for p53 induction of p53 by OP and provide support for the hypothesis that induction of p53 by OP in the normal cell line results from the activation of a DNA strand break recognition pathway. In contrast, the effect of PDTC on p53 appears to involve regulatory mechanisms distinct from those activated by DNA damage.

Inhibition of p53 activity by PDTC delays induction of p21^{WAF1} and cell cycle arrest induced by oxidative stress. To evaluate the functional consequences of p53 down-regulation by PDTC, we analyzed how exposure to PDTC affects the p53-dependent responses of MCF-7 cells to oxidative DNA damage by H_2O_2. In the absence of PDTC, H_2O_2 at 200 μM induces the accumulation of p53 (within 2 to 4 h), followed by induction of p21^{waf-1} and transient cell cycle arrest both in G_1 and in G_2/M, detected within 24 h by the presence of a significant decrease in the number of cells in S phase, correlated with an increase in the number of cells in G_1 and G_2/M fractions (44) (see also Fig. 6 and 7). Figure 1 showed that addition

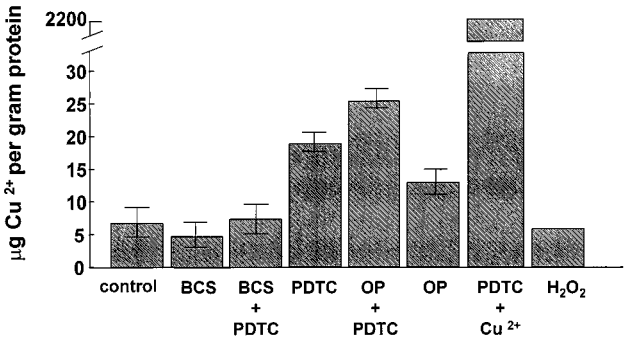

FIG. 3. Determination of the intracellular levels of copper in MCF-7 cells. MCF-7 cells were incubated for 2 h with 40 μM PDTC, 200 μM BCS, 200 μM OP, 40 μM $CuSO_4$, and the indicated combinations thereof. Cells were handled with chelex-treated solutions to minimize external contamination of metals and analyzed by electrothermal absorption spectrometry. The data are expressed as means ± standard error of the mean from three independent experiments.

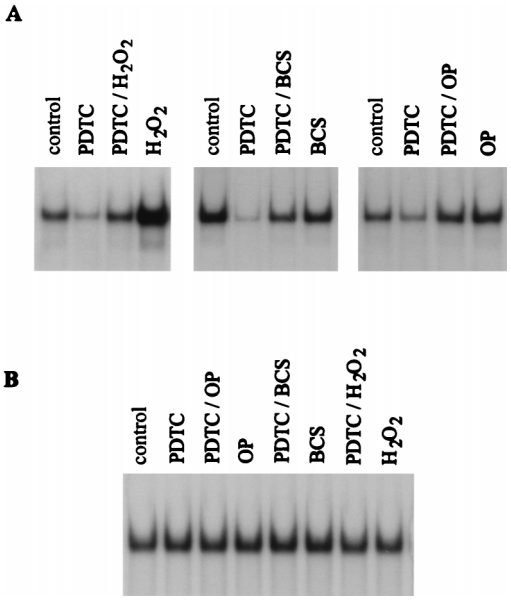

FIG. 4. Effect of metal chelators on the inhibition of p53 activity by PDTC. (A) Effect of metal chelators on the PDTC-suppressed DNA-binding activity of p53. MCF-7 cells were treated with 40 μM PDTC, 200 μM BCS, 200 μM OP, and the indicated combinations thereof for 2 h. Nuclear extracts were prepared, incubated with the ^{32}P-labelled p53^{CS} probe, and analyzed by EMSA. Only a section of the radiograph, containing the p53-DNA-PAb421 complexes, is shown. (B) Effect of metal chelators on the DNA-binding activity of the ubiquitous transcription factor Oct-1. The same nuclear extracts as described for Fig. 3A were incubated with a ^{32}P-labelled oligonucleotide, encompassing the Oct-1 consensus motif, and analyzed by EMSA. For details, see the legend to Fig. 2A. Only a section of the radiograph, containing the Oct-1-DNA complexes, is shown.

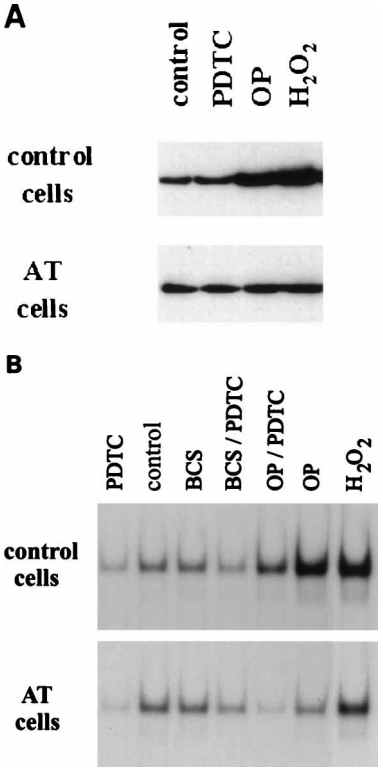

FIG. 5. DNA-binding activity of p53 in lymphoblastoid cells in response to metal chelation. (A) Control experiments showing levels of p53 protein in normal (non-AT) lymphoblastoid cells (IARC1104) and in lymphoblastoid cells derived from an AT patient (GM3189). (B) Analysis of DNA-binding activity. Cells were treated as described for Fig. 3A. Nuclear extracts were prepared and analyzed by Western blotting with DO-7 (A) or incubated with the p53^{CS} probe and analyzed by EMSA (B). Only a section of the radiographs, containing the p53-DNA-PAb421 complexes, is shown in panel B.

of PDTC was able to block the induction of p53 by H_2O_2. To determine whether this down-regulation was sufficient to completely prevent the p53-dependent response to H_2O_2, the cells were first exposed for 2 h to H_2O_2 and PDTC and the culture medium was then replaced by normal medium. The levels of p53 and p21^{waf-1} were monitored for up to 4 h after the medium change, and the cell cycle was analyzed at 6 and 24 h. The results of this experiment are shown in Fig. 6 and 7. First, with H_2O_2, accumulation of p53 was clearly detected after 2 h of exposure, but this accumulation was transient and p53 protein levels were back to almost basal levels 4 h after replacement of the culture medium. Induction of p53 was followed by an increase in the levels of p21^{waf-1} (Fig. 6) and by transient cell cycle arrest (Fig. 7). In the presence of PDTC and H_2O_2, p53 accumulation and p21^{waf-1} induction were prevented. However, an increase in the level of p21^{waf-1} was observed immediately after replacement of the culture medium containing both agents by fresh medium (Fig. 6), and this increase was followed by transient cell cycle arrest after 24 h (Fig. 7). Examination of the cell cycle at 6 h showed that the addition of PDTC induced a 2-h delay in the accumulation of cells in S phase (Fig. 7; compare the positions of the black arrowheads), consistent with the 2-h delay seen in the induction of p21^{waf-1}. Transient exposure (2 h) to either PDTC or OP alone resulted in a weak induction of p21^{waf-1} (Fig. 6), with no detectable effect on the cell cycle (data not shown). These data indicate that suppression of p53 DNA-binding activity by PDTC is reversed as soon as PDTC is removed from the culture medium. Restoration of p53 function results in transcriptional activation of p21^{waf-1}, followed by cell cycle arrest. This suggests that PDTC does not prevent the formation of DNA damage induced by H_2O_2 but prevents p53 becoming activated by DNA damage-dependent signals. This hypothesis is in agreement with the observation that PDTC does not prevent, and even increases, DNA strand break formation by oxidative stress (reference 35 and data not shown). Removal of PDTC allows p53 to again become receptive to signals generated by DNA damage-dependent pathways. These experiments, as well as those with OP presented in Fig. 5, argue for the existence of metal- and redox-dependent mechanisms regulating p53 func-

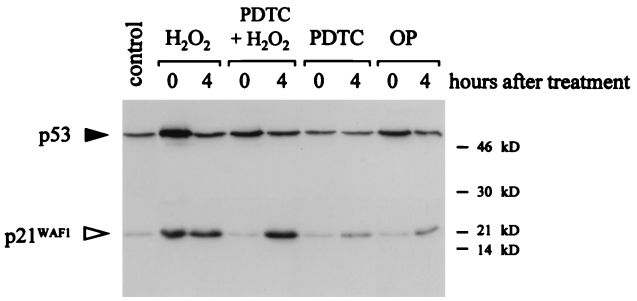

FIG. 6. Accumulation of p53 and p21^{WAF1} protein after treatment of cells with various metal chelators. MCF-7 cells were treated with metal chelators for 2 h as described for Fig. 3A and postincubated in drug-free medium for 0 or 4 h. The amount of p53 and p21^{WAF1} protein in nuclear extracts was determined by Western blot analysis. The black arrowhead indicates the position of p53 protein, and the white arrowhead indicates the position of p21^{WAF1} protein.

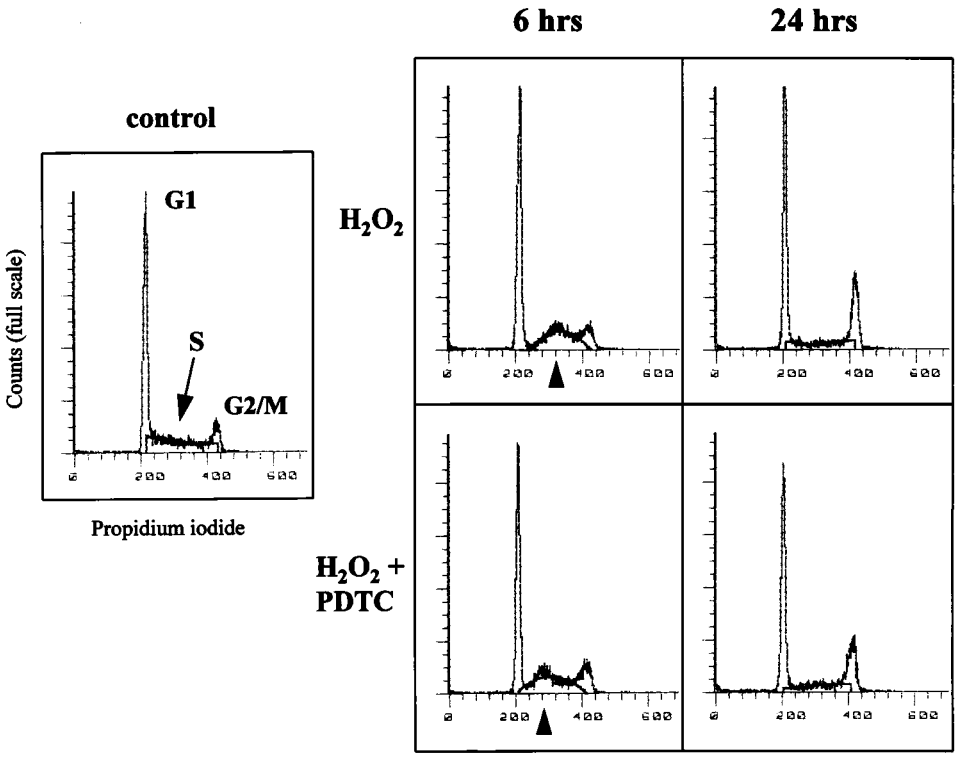

FIG. 7. Cell cycle distribution of MCF-7 cells after treatment with H_2O_2 and PDTC. MCF-7 cells were treated for 2 h with 200 μM H_2O_2 in the presence or absence of 40 μM PDTC. After replacement of the medium with drug-free medium, the cells were postincubated for the indicated times, trypsinized, RNase treated, labelled with propidium iodide, and analyzed with a Becton-Dickinson FACScan flow cytometer. Cell cycle distributions were calculated with cellFIT software. The results are displayed as histograms of cell number (counts) versus DNA content (propidium iodide). Arrowheads indicate the transient accumulation of cells in the S phase of the cell cycle. Coefficients of variation were ≤3.0%.

tion, which are distinct from the mechanisms by which p53 senses DNA damage.

DISCUSSION

Role of metals and redox factors in the allosteric model of p53. Induction of the sequence-specific transcriptional activity of p53 by genotoxic stress involves both quantitative and qualitative aspects, with rapid and transient protein accumulation within the nucleus and activation of its DNA-binding capacity. In normal cells, p53 exists in a "latent" form which can be activated to bind DNA by low levels of DNA-damaging agents, without any apparent increase in protein levels (23). This indicates that allosteric mechanisms play a role in the control of p53 activity. An allosteric model proposes that conversion from "latent" to "active" form requires the disruption of a negative regulatory interaction between the DNA-binding core and the C-terminal tail of p53, resulting in the unmasking of the DNA-binding surface of the protein. Typically, this process can be mimicked by neutralization of the C terminus of p53 with the monoclonal antibody PAb421 (21–23). There is also substantial in vitro evidence that the DNA-binding domain of p53 is flexible and can reversibly adopt distinct conformations that can be distinguished by reactivity with specific monoclonal antibodies (19, 30). The wild-type conformation is competent for DNA binding, whereas the so-called mutant conformation has lost DNA-binding capacity. In vitro, conversion from the wild-type to the mutant form can be induced by chelation of zinc and by oxidation of cysteines (16, 17). Both mechanisms are thought to affect the shape of the DNA-binding domain by disrupting the coordination of zinc, which plays an important structural role as a support for the DNA-binding surface of p53. The role of oxidation-reduction was further substantiated by Rainwater et al. (39), who showed that reduction of several conserved residues within the DNA-binding domain was essential for high-affinity binding to DNA and transactivation.

In this paper, we have used PDTC, a dithiocarbamate well known for its antioxidant effects in the pathway of activation of NF-κB and AP1 (29, 41), to test the hypothesis that redox modulation of p53 conformation may play a role in the control of p53 function. Our results show that PDTC down-regulates the DNA-binding activity of p53 by inducing a conformational switch that converts wild-type p53 to a partially mutant form. Why a proportion of p53 is apparently not affected by PDTC and remains in the wild-type form is unknown. Although p53 binds DNA as a tetramer, the protein can exist in several distinct oligomeric states and can also form complexes with multiple cellular proteins. It is possible that some of these complexes are resistant to the effect of PDTC.

Copper ions as modulators of p53 protein conformation. Since PDTC is widely considered a thiol antioxidant, we expected that it may favor the reduction of thiol groups in p53 and thus stabilize the molecule into the wild-type form, which is competent for DNA binding. That we observed the opposite effect led us to investigate whether PDTC could exert its effect indirectly, by modulating a cellular pathway which is important for the control of p53 conformation. In a recent paper, Nobel et al. (35) demonstrated that PDTC could exert opposite effects in different situations. Using thymocytes in culture, they showed that PDTC could bind trace amounts of copper in the culture medium with high affinity and mediate its intracellular

import and accumulation. Inside the cells, bound copper is apparently released into a usable pool, and accumulation of copper was quickly followed by a marked decrease in the level of reduced glutathione, an indicator of intracellular oxidative stress (35).

Our results with MCF-7 cells exposed to PDTC are compatible with those of Nobel et al. (35). We found that levels of intracellular copper were increased in MCF-7 cells exposed to PDTC and that blocking this increase with BCS, a chelator that does not cross plasma membranes, prevented down-regulation of p53 by PDTC. However, in contrast to Nobel et al. (35), we did not detect significant increase in intracellular oxidative stress: the ratio of oxidized to reduced glutathione was unchanged, and there was no increase in lipid peroxidation. Moreover, we did not observe any significant changes in the levels of zinc and iron, two other metals that may be chelated by PDTC.

These data suggest that down-regulation of p53 by PDTC resulted from a direct interference of copper ions with the p53 molecule. In a previous study, we have shown that a recombinant p53 polypeptide could specifically bind copper in the form of Cu^{1+} ions and that low levels of copper could disrupt p53 conformation and DNA-binding activity in vitro (18). The results reported here are consistent with these in vitro findings and confirm that copper can act as a regulator of p53 protein conformation and DNA-binding capacity in intact cells.

Copper has opposite effects on p53, depending upon the redox activity of copper chelates. Copper is an essential transition metal that plays a structural role in several transcription factors and a catalytic role in metalloenzymes such as copper-zinc superoxide dismutase (36). Inside cells, copper exists in several oxidation states and can jump from one redox state to another under physiological conditions. This redox cycling is responsible for both the catalytic and the toxic potential of this metal. Cu^{2+} directly interacts with several biological molecules and promotes their oxidation in an electron transfer reaction in which the reduced form of the metal, Cu^{1+}, is formed. In turn, Cu^{1+} reacts with molecular oxygen to form O_2^-, which dismutates to H_2O_2 and O_2, ultimately producing hydroxyl radicals and other reactive oxygen species. These active oxygen species can cause oxidative DNA damage and are responsible for the clastogenic potential of copper (2, 5, 49).

BCS is a specific chelator of copper in the Cu^{1+} form and has been shown to inhibit the clastogenic activity of copper by immobilizing copper in Cu^{1+} (26). In contrast, OP stimulates the redox activity of copper ions. The copper-OP complex behaves as a chemical endonuclease, stimulating internucleosomal DNA fragmentation, and exposure of cells to OP alone can stimulate DNA strand break damage by promoting the redox activity of endogenous copper (6). In MCF-7 cells, OP also increased the intracellular level of copper but had an effect opposite to that of PDTC on p53 DNA-binding activity. The effect of OP is consistent with induction of p53 via a DNA damage-dependent pathway. Indeed, OP did not stimulate p53 in lymphoblastoid cells from patients with AT, which are deficient for p53 induction by DNA strand break damage. In contrast, cells from AT patients showed similar responses to PDTC and BCS as control cells. These results indicate that elevation of intracellular copper levels per se is not sufficient for down-regulation of p53 and that copper may exert either stimulatory or inhibitory effects on p53 depending upon the redox activity. Whereas the stimulatory effect is dependent upon oxidative DNA damage, the inhibitory effect apparently operates through an unrelated pathway which is intact in cells from AT patients.

How copper affects p53 conformation and activity is a matter of speculation. Cu^{1+} preferentially forms tetrahedral Cu^{1+}-cysteinyl thiolate complexes, and in a previous paper we have proposed that copper could replace zinc within the DNA-binding domain of p53, disrupting the architecture of this domain (18). Another hypothesis is that copper may interact with other conserved cysteinyl residues in p53, such as Cys^{275} and Cys^{277}, which form a C-X-C motif common among Cu^{1+}-binding proteins. However, the thiol groups of these two cysteines are apparently pointed in opposite directions (7), and it is unlikely that this motif will participate in tetrahedral metal coordination. Further work is required to determine the exact site and geometry of the interaction between p53 and copper.

Does copper play a physiological role in the control of p53? Whether changes in copper levels can play a physiological role in the control of p53 remains to be determined. In this paper, we show that modulation of p53 activity by PDTC is reversible and that after removal of PDTC, p53 again becomes capable of being activated in response to DNA damage. Transient exposure to PDTC results in a delay in the p53-mediated cell cycle arrest induced by DNA damage. These data show that small and reversible changes in intracellular copper levels may act as a second-messenger system in a signalling pathway controlling p53 function. The concept that metal ions may act as intracellular second messengers is well established for calcium. It has recently been proposed that zinc may also behave as an information-carrying molecule in vivo (3). Given the similarities between zinc and copper and their capacity to interact with identical protein structures, it is tempting to speculate that these two metals can act as partners to regulate the activity of specific metal-binding proteins in a manner which is dependent upon redox conditions. Intracellular levels of zinc and copper are tightly controlled by a class of small inducible proteins, the metallothioneins, and changes in the levels of metallothioneins occur during cell cycle progression (34) as well as in several cancers (9). Furthermore, breakdown of the copper/zinc partnership may contribute to cancer. In many malignancies, levels of copper are significantly increased (27, 28). A human hereditary recessive disorder, Wilson's disease, is characterized by a perturbed copper metabolism. In patients with Wilson's disease, copper accumulates to high levels in liver cells, resulting in cytotoxicity (reviewed in reference 32). In the mouse genetic model of Wilson's disease, the LEC mouse, accumulation of copper in the liver results in hepatitis and hepatocellular carcinoma (47). The mechanisms underlying copper-induced carcinogenesis are, however, poorly understood, and further studies are needed to determine the role of p53 in this process. Interactions between p53 and copper/zinc provide a molecular basis for understanding how metals and redox changes cooperate to modulate p53 conformation and activity. Whether this modulation plays a role in the sensing by p53 of hypoxic stress and of genotoxic stress remains to be determined.

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