Cadmium Induces Conformational Modifications of Wild-type p53 and Suppresses p53 Response to DNA Damage in Cultured Cells*

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The p53 tumor suppressor protein is a transcription factor that binds DNA in a sequence-specific manner through a protein domain stabilized by the coordination of zinc within a tetrahedral cluster of three cysteine residues and one histidine residue. We show that cadmium, a metal that binds thiols with high affinity and substitutes for zinc in the cysteiny1 clusters of many proteins, inhibits the binding of recombinant, purified murine p53 to DNA. In human breast cancer MCF7 cells (expressing wild-type p53), exposure to cadmium (5–40 μM) disrupts native (wild-type) p53 conformation, inhibits DNA binding, and down-regulates transcriptional activation of a reporter gene. Cadmium at 10–30 μM impairs the p53 induction in response to DNA-damaging agents such as actinomycin D, methylmethane sulfonate, and hydrogen peroxide. Exposure to cadmium at 20 μM also suppresses the p53-dependent cell cycle arrest in G1 and G2/M phases induced by γ-irradiation. These observations indicate that cadmium at subtoxic levels impairs p53 function by inducing conformational changes in the wild-type protein. There is evidence that cadmium is carcinogenic to humans, in particular for lung and prostate, and cadmium is known to accumulate in several organs. This inhibition of p53 function could play a role in cadmium carcinogenicity.

The p53 protein is a tumor-suppressive transcription factor activated in response to multiple signals including radiation, genotoxic chemicals, hypoxia, depletion of ribonucleotides, and poisoning of the mitotic spindle. In most normal, nonexposed cells, p53 is a latent factor. Induction in response to stress involves nuclear accumulation (as a result of escape from mdm-2-mediated degradation and nuclear export) and conversion to an active form with high affinity for specific DNA sequences. Activation requires post-translational modifications at both the N and C terminus of the protein, including changes in phosphorylation, acetylation, and binding to heterologous proteins (1–5). Activated p53 controls several sets of genes to prevent the proliferation of cells under stress conditions. Genes trans-activated by p53 include inhibitors of cell cycle progression in G1 and G2 (p21waf1, 14-3-3σ, GADD 45), regulators of apoptosis (APO1-Fas/CD95, Bax-1, KILLER/DR5), and genes involved in the metabolism of reactive oxygen species (such as PIG-3, PIG-6, and PIG-12) that may play a role in induction of apoptosis (6, 7). p53 also represses a number of promoters and regulates transcription, replication, and DNA repair through interaction with proteins such as RP-A and components of TFIIID and TFIIH complexes (for recent reviews see Refs. 4 and 8–11).

High affinity binding of p53 to specific DNA sequences is mediated by a conformation-sensitive structure in the central portion of the protein (residues 102–292) (12). The structure of the DNA-binding domain consists of two β-sheets supporting a loop-sheet-helix motif (that interacts with the major groove of DNA) and a loop-helix motif (L2/L3, that interacts with the minor groove). L2/L3 is stabilized by tetrahedric coordination of zinc by residues Cys176, His179, Cys238, and Cys242 (13). Folded and unfolded forms of human wild-type p53 are distinguishable by their reactivity with the conformation-specific monoclonal antibodies PAb1620 (folded form, often termed “wild-type” conformation) and PAb240 (unfolded form, often termed “mutant” conformation).

The folding of the DNA-binding domain is sensitive to metal substitution and to oxido-reduction in vitro and in intact cells. Removal of zinc by chelation reversibly alters p53 conformation, with loss of DNA binding capacity (5, 14–16). Furthermore, metals such as copper, cadmium, or mercury induce p53 to adopt a PAb240- phenotype in vitro (17,18). These observations raise the possibility that exposure to toxic metals and perturbation of the physiological metal supply may affect p53 function in vivo.

Metals such as cadmium, chromium, nickel, and arsenic are classified in group 1 of the International Agency for Research on Cancer categories of carcinogens (carcinogenic to humans; for reviews, see Refs. 19–21). Cadmium is chemically close to zinc and binds with high affinity within the tetrahedral zinc-binding domains of several metalloproteins in vitro (22–24). Cadmium is a widespread environmental pollutant that is also present in tobacco smoke (1–3 μg/cigarette). Smoking, together with occupation, are the major sources of human exposure. Cadmium is absorbed by inhalation and ingestion and has a very long biological half-life (>25 years). Epidemiological studies have identified lung, prostate, and, to a lesser extent, kidney and stomach as primary targets for cadmium-induced tumorigenesis (21). In exposed industrial workers, cadmium accumulates in the kidneys (100–400 μg/g, wet weight) and liver (20–100 μg/g, wet weight), at levels that are 5–9 times higher than those of unexposed workers (25, 26). The kidneys and liver express high levels of metallothioneines, a class of stress response proteins that bind and detoxify cadmium.

The mechanisms of cadmium carcinogenesis are poorly understood. In vitro, at concentrations between 0.1 and 10 mmol, cadmium is cytotoxic and induces radical-dependent DNA damage (27, 28). However, compared with other carcinogenic metals, cadmium is a weak mutagen (29). At lower concentrations
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(1–100 μmol), cadmium binds to proteins, decreases DNA repair (30, 31), activates protein degradation, up-regulates cytokines and proto-oncogenes such as c-fos, c-jun, and c-myc (32, 33), and induces the expression of metallothioneins (34). Thus, cadmium carcinogenicity may involve multiple factors, including up-regulation of mitogenic signals and interference with DNA repair (for a review, see Ref. 19).

In this study, we have examined the effects of cadmium on p53 protein conformation, DNA binding, and transcriptional activity. Using the breast carcinoma MCF7 cell line, which expresses high levels of wild-type p53, we show that cadmium at subtoxic concentrations (10–30 μM) perturbs the folding of p53, disrupts DNA binding, impairs p53 induction by DNA-damaging agents, inhibits transactivation of a reporter gene and of target genes such as p21WAF1, and prevents cell cycle arrest in response to γ-irradiation. Based on these results, we propose that cadmium may inactivate wild-type p53 by altering metal-dependent folding and that this effect may contribute to cadmium carcinogenesis.

EXPERIMENTAL PROCEDURES

Purified Wild-type Recombinant p53—Murine p53 was produced in SF9-infected cells using a baculovirus expression system and was purified in buffers depleted of ion transition metals (“metal-free buffers” as described previously (5)). Metal-free buffers were prepared by incubation of DNA-binding reaction solutions with chelating resin (10% v/v) for 1 h at 4 °C and used immediately (Chelex-100, Sigma).

Cell Culture and Treatment—The human breast carcinoma cell line MCF7, expressing high levels of wild-type p53, was cultured at 37 °C under 10% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (PAA, Linz, Austria). Murine 10.1 fibroblasts (p53-deficient) were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (35). With the exception of the irradiation experiments, cells were plated in 6-cm Petri dishes and drug-treated at 70–80% confluency. A stock solution of CdCl2 (0.5 M) was prepared in 10 mM Tris, pH 7.4, and diluted for cell treatment. In case of pretreatment with cadmium, cells were exposed to CdCl2 (30 min or 2 h) before addition to the culture medium of actinomycin D (Act D; 2.5 μg/ml), methylnethane sulfonate (0.5 mM), or H2O2 (100 μM). All chemicals were from Sigma. Subconfluent MCF7 cells, plated in 75T flasks, were exposed to ionizing radiation by treatment in a β-irradiator (αTec) for an appropriate length of time to deliver the preselected dose of 5 Gy.

RNA Isolation and Northern Blot Analysis of Metallothionein (MT)-IIA mRNA—MCF7 cells were washed in sterile PBS, and total RNA was isolated using Trizol reagent as described by the manufacturer (Life Technologies, Inc.). Ten μg of total RNA were resolved on 1% agarose-formaldehyde gel, blotted onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) by capillary transfer, and UV-cross-linked (Stratalinker 1800, Stratagene, La Jolla, CA). The HindIII-3Kb cDNA fragment from pMTHIA-BP24 plasmid (36), the 1.3-kilobase pair p53 human cDNA probe, (HP 119–2, Oncogene Science Inc., Manhasset, NY), and a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe (CLONTECH, Palo-Alto, CA), were labeled by random priming with [32P]dCTP (RPN1606 Megaprime DNA labeling system; Amer- sham Pharmacia Biotech). Membranes were hybridized in CHURCH buffer (7% SDS, 1% bovine serum albumin, 0.5 nM NaHPO4) for 16 h at 65 °C and then washed twice in three different buffers (10 min in 2× SSC, 0.1% SDS at room temperature; 30 min in 2× SSC, 1% SDS at 65 °C; 5 min in 0.1× SSC at room temperature). Autoradiography was performed using Biomax MR film (Eastman Kodak Co.) at ~80 °C.

Cytosplasmic and Nuclear Protein Extractions—Cells were washed in PBS and collected by scraping. Cytosplasmic and nuclear extracts were prepared as described in Ref. 16. Briefly, cells were lysed in buffer A (20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40). After centrifugation, supernatants were kept as cytoplasmic extracts. Nuclear proteins were obtained by extraction of the pellet in buffer B (same as buffer A but with 0.5 M instead of 10 mM NaCl) in the presence of a mixture of protease inhibitors: 0.5 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 0.7 mg/ml pepstatin (all from Sigma). Protein contents were quantified by the Lowry method.

Detection of p53 and p21WAF1 Proteins by Western Blot Analysis— Nuclear protein extracts (30 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis (250 μg/ml; Pierce) was used as the second antibody, followed by ECL detection, as specified by the manufacturer (Amersham Pharmacia Biotech).

DNA-binding Assays—The protocols for DNA-binding assays were described earlier (16, 37). Briefly, the p53 consensus binding sequence p53CS (5′-GGACATGCCGCGGATGGTCC-3′) (5) and an oligonucleotide containing the Oct-1 binding sequence (underlined, 5′-GACCA CCGGTGAAATTTGATTTCAATAAATA-3′) (37) were end-labeled with ~3000Ci/mmol [γ-32P]ATP (Amersham Pharmacia Biotech). DNA binding experiments were performed for 30 min at room temperature. Murine recombinant protein (100 ng) was incubated with 0.5 ng of labeled p53CS (in 10 mM dithiothreitol, 5 μg of bovine serum albumin, 140 mM NaCl, 20 mM HEPES (pH 7.6), 20% glycerol, 0.1% Nonidet P-40) in the presence of herring sperm DNA (2.5 ng) as nonspecific competitor (43). The protocol was as follows for DNA-binding using nuclear extracts (46 μg) with the following modifications: 1) the concentration of competitor (herring sperm DNA) was increased to 2.2 μg; and 2) experiments were performed in the presence of 4 μM dithiothreitol. All experiments included PAB421 (100 ng) (OP30, Oncogene Science), a monoclonal antibody that stabilizes and supershifts p53-DNA complexes. With cellular extracts, no specific binding to DNA was detectable in the absence of PAB421. After incubation, DNA-p53 protein PAB421 complexes were resolved on a 4% nondenaturing polyacrylamide gel electrophoresis in 1× TBE for 2–3 h at 120 V. Gels were then fixed, dried, and exposed to Kodak x-ray films at ~80 °C. Control experiments using a mutant p53 consensus sequence, as well as competition experiments using unlabeled p53cs, were performed to demonstrate the specificity of binding.

Immunocytochemistry for p53—MCF7 cells were cultured in eight-chamber polystyrene tissue culture slides (Becton Dickinson, Mountain View, CA) until subconfluent and exposed to CdCl2 for 4 h. Cells were then washed in PBS, fixed in 1:1 (v/v) cold methanol/acetic acid for 4 min, incubated for 1 h in PBS/Nonidet P-40 0.1% containing 5% bovine serum albumin, and incubated for 1 h at room temperature with the anti-p53 monoclonal antibody PAb1801 (1 μg/ml, OP90, Oncogene Science). After five washings in PBS/Nonidet P-40 0.1%, fixed antibodies were detected with goat anti-mouse immunoglobulin G (1:300 dilution; Pierce), followed by diaminobenzidine staining using diaminobenzidine peroxidase enhanced with nickel (Vector Laboratories, Burlingam, CA).

Conformation-specific Immunoprecipitation of p53—MCF7 cells were washed in PBS, lysed 10 min on ice in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.5% Nonidet P-40, with protease inhibitors as above), scraped, and kept on ice for another 10 min prior to 5-min centrifugation at 15,000 × g at 4 °C. Supernatants were preclreated by incubation with 1 μg of a non-anti-p53 antibody (PAB416, specific for large T antigen of Simian virus 40 (SV40), DP29, Oncogene Science) for 15 min at 4 °C with shaking followed by incubation with 10% (v/v) of Staphylococcus aureus protein A suspension (50 μl) (Sigma) for 15 min and by 5-min centrifugation (15,000 × g) at 4 °C. Supernatants were aliquoted for immunoprecipitation with monoclonal antibodies PAB1820 (specific for the wild-type, folded form, OP33), PAB240 (specific for the mutant, unfolded form, OP29), PAB421 (which recognizes both forms, OP30), and PAB416 (as negative control, DP29) (all from Oncogene Science). Immune complexes were collected using S. aureus protein A suspension and washed five times in immunoprecipitation buffer. Precipitates were then denatured in Laemmli buffer and analyzed by Western blot experiments using the rabbit polyclonal anti-p53 antibody CM-1 (1:1000 dilution; Novocastra, Newcastle, UK) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (250 ng/ ml) as secondary antibody. After exposure to ECL (Amersham Pharmacia Biotech), proteins were quantified using the Bio-Rad densitometer.

Transfections and β-Galactosidase Assays—Murine 10.1 cells were co-transfected by the calcium phosphate method with the reporter plasmid pRGC3FosLucZ, containing the p53 binding site located in the ribosomal gene cluster (RGC-βgal) (39), and p53pcDNA, expressing full-length human p53 cDNA located under a cytomegalovirus promoter. Medium was changed 16 h later, and cells were further cultured...
RESULTS

Cadmium Inhibits Specific DNA Binding by Recombinant Wild-type p53—Previous studies using in vitro translated murine p53 have shown that cadmium induces conformational changes in p53 with loss of the immunological, wild-type phenotype (reactive with PAb1620) (17). To better characterize the effect of cadmium on the p53 protein, we incubated purified, baculovirus-produced murine wild-type p53 with CdCl₂ or ZnCl₂ and analyzed its DNA-binding capacity by electrophoretic mobility shift assay (EMSA). Fig. 1 shows that divalent metal ions are required for DNA binding, since no activity was observed in buffers depleted of transition metal (treated with Chelex resin) (lane 1). Binding was restored in non-Chelex buffers containing trace amounts of divalent metals (lane 2). Binding was increased when the protein was incubated in 20 μM ZnCl₂ prior to EMSA (lane 3). In contrast, incubation with CdCl₂ (2–16 μM) induced a dose-dependent inhibition of DNA binding activity (lanes 4–7). This result confirms the role of zinc in DNA binding competence and shows that cadmium inhibits DNA binding, consistent with the hypothesis that it may compete with zinc in binding to reactive cysteines within the DNA-binding domain of p53.

Effect of CdCl₂ on Expression of p53 and of Metallothioneins—The MCF7 breast carcinoma cell line expresses high levels of wild-type p53 and is commonly used as a cellular model to assess p53 functions. Northern blot analysis of cells exposed to 10 μM CdCl₂ showed a strong, time-dependent, increase in the expression of MT-IIA, a metal-binding protein specifically induced by cadmium. Increased mRNA expression was already detectable after 1 h and reached a plateau (50-fold) after 6–12 h. In contrast, levels of p53 mRNA remained unchanged after up to 24 h of treatment (Fig. 2). These results indicate that cadmium was rapidly taken up by MCF7 cells and induced the expression of a metalloregulated gene but did not affect the level of p53 mRNA.

Down-regulation of p53 DNA Binding Activity in MCF7 Treated with CdCl₂—Cadmium is a potent cytotoxic agent known to induce oxidative stress in cultured cells (40). Trypan blue exclusion tests showed that the percentage of surviving MCF7 cells after 24 h of culture in the presence of cadmium was 93, 50, and 20% at, respectively, 10, 20, and 30 μM CdCl₂. Cytotoxicity was time-dependent, and no significant cell death was observed at up to 8 h of incubation. To evaluate the long term effects of short exposures to cadmium, cells were incubated for 4 h with CdCl₂ and then further cultured for 20 h in CdCl₂-free culture medium. Under these conditions, no significant cytotoxicity was shown at 10 μM CdCl₂, and up to 80% of cells survived with 20 μM CdCl₂. Nevertheless, treatment with 30 μM CdCl₂ still induced a sharp drop in cell viability (by 75%) (data not shown). Unless otherwise stated, in all experiments reported here, cells were exposed to CdCl₂ for 4 h and immediately harvested in conditions where no significant decrease in viability was observed.

Western blot analysis of p53 levels in cells exposed to CdCl₂ showed that cadmium induced a dose-dependent accumulation of p53 after 4 h of exposure, with a maximum increase of 2.3-fold at 20 μM, followed by a decrease at higher doses (Fig. 2). Since no effect was seen on the p53 mRNA (Fig. 2), this accumulation may result from protein stabilization. DNA binding activity also showed a biphasic dose response, with first a slight increase at 10 μM, followed by a marked inhibition at 20 μM and above (Fig. 2A, middle panel). There was no significant change in the level of binding of Oct-1, a ubiquitous, constitutively expressed transcription factor used as a control (Fig. 2A, lower panel). These results indicate that cadmium exerted complex effects on p53 in intact cells, with accumulation of the protein (compared with untreated cells) and DNA binding inhibition (at concentrations of CdCl₂ equal to or greater than 20 μM). At 10 μM, cadmium increased both p53 levels and DNA binding activity, consistent with the notion that this metal may induce oxidative DNA damage. However, at higher concentrations (20 μM and above), cadmium downregulated DNA binding activity even if p53 protein levels remained higher than in nontreated cells. Binding of the control...
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transcription factor Oct-1 shows that inhibition of p53 did not correspond to a general toxic effect on DNA-binding proteins. Similar results were obtained with other cell lines expressing wild-type p53 (3T3 mouse fibroblasts and A549 human lung carcinoma cells) (data not shown).

DNA binding of p53 in cell extracts requires the addition of PAB421, a monoclonal antibody that binds to the C terminus of the protein and stabilizes its activity (see "Experimental Procedures"). To determine if cadmium may inhibit DNA binding by interfering with the binding of PAB421, the p53 protein was immunoprecipitated from control and cadmium-treated cells, and p53 levels were determined by Western blotting. Quantitative analysis revealed variations of less than 10%, indicating that DNA binding inhibition by cadmium was not due to impaired reactivity of p53 with PAB421.

Nuclear Accumulation of p53 in Cells Exposed to CdCl₂—To determine if inhibition of DNA binding activity by cadmium was the result of cytoplasmic sequestration, we analyzed p53 localization in MCF7 cells by immunostaining. In some reports, it has been reported that growth stimulation of serum-starved MCF7 cells induces a protein synthesis-dependent cytoplasmic sequestration of p53 protein (41, 42). However, this particular feature has not been found by others (43–45). In our experiments, p53 was found to localize essentially, if not exclusively, in the nucleus of exponentially growing MCF7 cells (Fig. 3B). In untreated cells, staining with the anti-p53 antibody PAB1801 was heterogeneous, with most cells showing a very low level of reactivity and a small number of cells clearly positive for nuclear staining. As positive control, exposure to Act D, a topoisomerase II inhibitor that is a strong inducer of p53, induced nuclear accumulation of p53 in almost every cell. After 4 h of exposure to 30 μM CdCl₂, p53 was also present in the nucleus of most cells. Therefore, the loss of DNA binding activity shown in Fig. 3A cannot be explained by cytoplasmic sequestration of p53 in cells exposed to cadmium.

Effects of CdCl₂ on p53 Protein Immunological Phenotype—To determine if conformational changes could account for down-regulation of p53 DNA binding activity by cadmium, we analyzed the reactivity of p53 in MCF7 cells using conformation-specific monoclonal antibodies PAB1620 and PAB240. In nontreated cells, p53 adopted a wild-type conformation, reactive with PAB1620 and PAB240 (specific for mutant, unfolded conformation), PAB1801 (specific for wild-type, folded conformation), and PAB421 (which reacts with both conformers). PAB416 (specific for SV40 LT) was used as a negative control. Immunoprecipitates were analyzed by Western blot with the rabbit antibody CM-1. Black arrows, p53. A minor, p53-related band is also detected at 42 kDa.

Reversibility of the Effects of CdCl₂ on p53 DNA Binding Activity—To determine if the effects of cadmium on p53 were reversible, MCF7 cells were first exposed to CdCl₂ for 4 h (30 μM), and the culture medium was then replaced by fresh, CdCl₂-free medium and further cultured for 12–24 h. Fig. 4 shows that removal of CdCl₂ from medium resulted in partial recovery of p53 DNA binding activity after 12 h (lane 4) or 24 h (lane 7). In contrast, DNA binding activity remained undetectable in cells that were continuously cultured in the presence of CdCl₂ for the same periods of time (lanes 5 and 8). It was noted that culture of MCF7 cells for 12 or 24 h in the presence of CdCl₂ at 30 μM affected the survival MCF7 cells (see above). This cytotoxicity may explain the strong decrease of p53 protein levels seen in lanes 5 and 8 (Fig. 4B) and the concomitant absence of DNA binding activity.

Cadmium Prevents p53 Activation by DNA-damaging Agents—The results presented above suggest that cadmium may disrupt p53 protein conformation, thereby abrogating its tumor-suppressive functions in cells exposed to other forms of genotoxic stress. To test this hypothesis, we have analyzed the induction of p53 by Act D in the presence of CdCl₂. MCF7 cells were exposed for 2 h to CdCl₂ (5–30 μM) before the addition of Act D to the culture medium (2.5 ng/ml) and were further cultured in the presence of both agents for 4 h (Fig. 5). In the...
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Fig. 5. Effect of cadmium on activation of p53 DNA binding by Act D. A, MCF7 cells were cultured for 2 h with CdCl₂ at concentrations as indicated, and Act D (2.5 ng/ml) was added for another 4 h. Levels of p53 in nuclear extracts were then analyzed by Western blot. DNA binding of p53 and of Oct-1 was analyzed by EMSA. A, densitometric analysis of the effect of 10 μM CdCl₂ on p53 DNA binding activity. Average and S.D. for autoradiograms of three independent experiments such as the one in A were analyzed. C, immunoprecipitation of p53 in cells exposed to CdCl₂ and Act D. MCF7 cells were exposed to 10 μM CdCl₂ for 30 min prior to treatment with Act D. p53 was immunoprecipitated and analyzed as described in the legend to Fig. 3C. Filled arrowheads, p53. Several minor, slowly migrating bands are also detected. They may correspond to ubiquitinated forms of p53 (64).

absence of CdCl₂, Act D induced a 3-fold accumulation of p53 protein and a 10-fold activation of DNA-binding capacity. CdCl₂ induced a dose-dependent inhibition of the effect of Act D on p53 DNA binding activity (Fig. 5A), consistent with data presented in Figs. 3 and 4.

To examine whether cadmium at low, nontoxic doses could also impair p53 activation, MCF7 cells were pretreated for 30 min with 10 μM CdCl₂ before the addition of Act D for 4 h. DNA binding activity was analyzed by EMSA and quantified by densitometric analysis of autoradiograms (Fig. 5B), and p53 conformation of was analyzed by immunoprecipitation (Fig. 5C). In the absence of Act D, CdCl₂ induced a small but significant increase in p53 DNA binding activity (2.28 ± 0.26-fold). In the presence of Act D, CdCl₂ (at 10 μM) decreased by 40% the p53 activation triggered by Act D alone (Fig. 5B). This effect was correlated with a change in the conformation of a fraction of the p53 molecules as detected immunoprecipitation with conformational specific antibodies (Fig. 5C). CdCl₂ treatment at 10 μM induced a small proportion of p53 to become reactive with PAH240 and this effect was more marked in cells exposed to both CdCl₂ and Act D. Overall, these data indicate that CdCl₂ at 10 μM had a clear inhibitory effect on p53 activation by the DNA-damaging agent Act D. Similar results were observed with two other DNA-damaging agents, methylethanesulfonate (an alkylating agent) and hydrogen peroxide (H₂O₂) (data not shown).

Cadmium Impairs Transactivation of Target Genes by p53—To determine if CdCl₂ may impair p53 protein function, we have co-transfected human wild-type p53 and the p53-dependent reporter construct pRGČΔFosLacZ into the p53-null mouse fibroblast cell line BalbC10.1. Twenty h after transfection, CdCl₂ was added to the culture medium, and β-galactosidase activity was measured 12 h later. Fig. 6A shows that the induction of CdCl₂ at the noncytotoxic dose of 10 μM reduced β-galactosidase activity by 65% in cells transfected with p53 (p < 0.002). In the absence of p53, CdCl₂ at 10 μM had no detectable effects on basal β-galactosidase activity. A stronger decrease (85%) was seen with 30 μM CdCl₂. However, part of this effect may reflect the cytotoxicity of exposure for 20 h to CdCl₂ at 30 μM.

The expression of p21<sup>wt-1</sup> protein in MCF7 cells exposed to CdCl₂ was subsequently analyzed (Fig. 6B). Although p21<sup>wt-1</sup> is transcriptionally regulated by several factors other than p53, there is good evidence that p21<sup>wt-1</sup> expression in MCF7 cells is essentially p53-dependent (46). Levels of p21<sup>wt-1</sup> protein increased at 10 μM CdCl₂ but decreased at 30 μM. This observation is consistent with the biphasic effect shown in Fig. 3A. Preincubation of cells with CdCl₂ at 30 μM prevented the induction of p21 by either of the DNA-damaging agents H₂O₂ and methylethanesulfonate. Moreover, the extent of p21<sup>wt-1</sup> induction was reduced by 49 ± 21% in cells exposed to these agents in the presence of 10 μM CdCl₂ (p < 0.001). Similar results were obtained for Mdm-2, another transcriptional target of p53 (data not shown).

Cadmium Inhibits p53-dependent G<sub>1</sub> Arrest after γ-Irradiation—In MCF7 cells, activation of p53 by ionizing radiation induces in cell cycle arrest in both the G₁ and G<sub>S</sub>/M phases (47). To determine whether the down-regulation of p53 activity by cadmium resulted in a disruption of cell cycle control, cells were exposed to cadmium prior to γ-irradiation, and cell cycle distribution was analyzed by flow cytometry after labeling with propidium iodide (Fig. 7 and Table I). In nonirradiated cells, CdCl₂ did not alter cell cycle distribution at 10 or 20 μM, but a marked increase in the sub-G₁ fraction corresponding to cell debris (7%) was observed at 30 μM. However, even at the latter concentration, the overall distribution of cells in G₁, S, and G<sub>S</sub>/M phases was essentially unchanged, suggesting that cad-
Irradiation at 5 Gy induced a cell cycle delay in G1, detected as increased G1 phase, a consequence of the toxicity of cadmium. 5 Gy corresponds to irradiated cells exposed to CdCl2 at concentrations as indicated (see also the legend to Fig. 7).

**TABLE I**

| Phase | 0 μM CdCl2 | 10 μM CdCl2 | 20 μM CdCl2 | 30 μM CdCl2 |
|-------|------------|-------------|-------------|-------------|
| Control | 5 Gy | Control | 5 Gy | Control | 5 Gy |
| Sub-G1 | % | % | % | % |
| G1/G0 | 1 | 1 | 1 | 1 | 7 | 8 |
| S | 61 | 69 | 60 | 67 | 64 | 64 | 57 | 56 |
| G2/M | 28 | 15 | 29 | 20 | 27 | 26 | 31 | 30 |
| Control | 5 Gy | Control | 5 Gy | Control | 5 Gy | Control | 5 Gy |
| Sub-G1 | 10 | 10 | 12 | 8 | 9 | 5 | 6 |

These data indicate that MCF7 cells exposed to cadmium show impaired cell cycle arrest in response to γ-radiation, a well known p53-dependent response, and that this effect is not a consequence of the toxicity of cadmium.

**DISCUSSION**

Zinc is essential for correct folding of wild-type p53. The DNA-binding domain contains a tetrahedrally coordinated zinc that stabilizes two loops at the DNA-binding surface of the protein (13). Several in vitro studies have shown that metal chelation abolishes binding of p53 to specific DNA (12, 17, 37, 48). In addition, metal chelation increases oxidation of cysteines in p53, indicating that zinc binding is not purely structural but also controls the sensitivity of p53 to oxidation-reduction (17, 49). Reduction of cysteines stimulates p53 DNA-binding (5, 15), and Ref-1, a protein that regulates the redox state of several transcription factors, is a potent activator of p53 (51). In vitro, the conformational and DNA-binding capacity of p53 are altered by incubation with metals chemically close to zinc, such as cadmium and copper, but not with cobalt, magnesium, manganese, or iron (17, 52). These observations have led to the suggestion that specific metals and redox factors may affect the fine tuning of p53 and participate in the physiological control of p53 functions (5, 16, 51, 53).

We show here that sequence-specific DNA binding of p53 in vitro is decreased by cadmium in a dose-dependent manner. In MCF7 cells, transient exposure (4 h) to cadmium at 20 μM and above induced a change in p53 conformation (to the unfolded, PAb240+ form) with loss of DNA binding and transcriptional activity. These results are consistent with this idea that cadmium perturbs the folding of p53 in a direct or indirect manner.

The binding affinity of cadmium to cysteine thiolate clusters in zinc finger proteins is 2–3 orders of magnitude higher than that of zinc (22, 24, 57). Consistent with this notion, we found that the effect of cadmium on p53 was not reversed by the addition of excess ZnCl2 (up to 25-fold) to cadmium-treated MCF7 cells (data not shown). Therefore, although our data do not provide a formal proof that cadmium can displace zinc from native p53, our results are consistent with the idea that cadmium perturbs the metal-dependent folding of the DNA-binding domain.

In MCF7 cells, cadmium exerts complex, biphasic effects on p53 protein levels and DNA binding activity. At low concentrations (up to 10 μM), cadmium alone induces a small (2–3-fold) but reproducible accumulation of p53 protein, correlated with slightly enhanced DNA binding activity (2.28 ± 0.26-fold). This effect may be due to p53 protein stabilization by low levels of oxidative DNA-damage induced by cadmium. However, increasing the concentration of cadmium does not result in higher levels of p53 protein activation. In contrast, it significantly decreases p53 DNA binding activity (at 20 μM) and protein levels (at 40 μM). Furthermore, inhibition of p53 activity correlates with a change in protein conformation, with loss of PAb1620 reactivity (wild type-specific) and acquisition of the PAb240-positive phenotype (Fig. 3C). Along with the observation that cadmium does not prevent p53 localization in the nucleus (Fig. 3B), these data indicate that cadmium inhibits
p53 by turning it into an inactive, “mutant-like,” form.

Cadmium at 30 μM induces total inhibition of p53 protein activation in response to DNA-damaging agents such as Act D, methylmethane sulfonate, or H2O2. This inhibition resulted in a loss of transcriptional activation of several p53 target genes including p21Waf1/Cip1. Moreover, cadmium at nontoxic concentrations (10 μM) is sufficient to significantly reduce (by about 40%) the extent of p53 induction by DNA-damaging agents and therefore to perturb the response of p53 to DNA damage.

The apparent contradiction between the effects of cadmium at 10 and 30 μM may be resolved by considering that cadmium has two opposite effects on p53, with first protein stabilization as a result of generation of DNA damage by low doses of cadmium and, second, direct inhibition of p53 protein by metal substitution and conformational modifications at higher doses of cadmium. The level of p53 DNA binding activity detected in the presence of cadmium would thus depend upon a subtle balance between these two mechanisms.

Inhibition of p53 DNA binding activity by cadmium has important functional consequences in cultured cells. First, cadmium reduces p53-dependent transactivation of reporter or endogenous target genes. Second, cadmium prevents the cell cycle arrest induced by low doses of γ-irradiation in MCF7 cells, suggesting that cadmium can effectively suppress p53 protein function. Cells exposed to cadmium thus behave in a manner analogous to p53-deficient cells that retain the capacity to proliferate after exposure to DNA-damaging agents. A similar hypothesis has been proposed in the case of excess production of nitric oxide, which also induces conformational and functional changes in wild-type p53 (14). Impairment of p53 function by cadmium may contribute to decrease the cell capacity to respond to the DNA damage induced by other carcinogens, thereby increasing the likelihood of acquiring mutations leading to cancer.

Cadmium is highly toxic in most biological systems and has a very long biological half-life (about 25 years in humans (20)). Therefore, it is essential to consider whether the concentrations of cadmium used in our experiments are compatible with those that occur in target cells of exposed organisms. After exposure to cadmium, most of the intracellular pool of cadmium is bound to MTs, a class of inducible, metal-binding proteins that sequester cadmium and protect cells from its toxic effect. However, experiments with MT-I and -II knockout mice showed that cadmium also accumulates to high levels in the absence of MT. In MT-deficient mice, CdCl2 injected subcutaneously at 30 μg/kg accumulates in liver cells within 3–6 h at up to 20–25 μg/g of fresh tissue (58). These levels may correspond to intracellular concentrations 3–10-fold higher than those used in the present study. Although this dose of cadmium was toxic in MT-deficient mice, it produced only mild hepatotoxicity in control mice. Concentrations of cadmium of up to 25 μM are well tolerated in many cultured cell lines (30). Therefore, the effects reported here are compatible with concentrations of cadmium that are not lethal and can occur in biological systems after acute or chronic exposure.

Alteration of p53 protein conformation by cadmium was described previously, using in vitro translated p53, with concentrations of CdCl2 of 50–100 μM (17). In our study, we show that much lower concentrations of cadmium (10–30 μM) are able to alter p53 conformation and function in intact cells. This is the first report that a metal compound can inactivate p53 at doses compatible with biological effects.

In 1995, Zheng et al. (58) reported that cadmium could increase p53 mRNA levels in liver cells of mice injected with CdCl2. We did not observe such an effect in cultured MCF7 cells. It is important to note that induction of p53 mRNA was observed as a late event (after 6–12 h) in mice receiving a high, hepatotoxic dose of cadmium. Therefore, it is possible that elevated p53 mRNA may represent a response to cell damage rather than a direct effect of cadmium on p53 gene expression.

The mechanism of p53 inactivation described here may account for some of the unexplained properties of cadmium as a carcinogen. Indeed, cadmium is a weak genotoxic agent compared with metals such as copper, iron, nickel, and chromium. Therefore, mechanisms other than direct genotoxicity have been proposed to explain cadmium carcinogenesis (59). Exposure to cadmium enhances the persistence of DNA lesions induced by mutagens such as benzo(a)pyrene and methylmethane sulfonate in human cells, suggesting that cadmium may inhibit DNA repair. Recently, Daily and Hartwig (30) have shown that cadmium, as well as nickel, inhibits the repair of DNA damage after irradiation. These authors propose that cadmium may either inactivate repair enzymes directly, for example by reaction with a histidine or cysteine residue, or compete with and displace essential metal ions, a hypothesis compatible with the results presented here. Inhibition of p53 function may explain the persistence of DNA lesions in cells exposed to both carcinogens and cadmium. According to this model, cadmium would not act as a conventional mutagen but rather as an indirect carcinogen that sensitizes cells to the genotoxic effects of other carcinogens by switching off essential components of cell cycle control and DNA repair pathways involving p53.

Cadmium exerts complex effects on the growth and survival of normal and cancer cells. The metal was shown to induce apoptosis or necrosis in some cells and tissues and to reduce the growth and metastasis of human lung carcinoma xenografts in nude mice (60). In contrast, cadmium was shown to inhibit apoptosis induced by DNA-damaging metals such as chromium (61). These observations suggest that the sensitivity to cadmium may vary from one cell type to another and that some cancer cells may be hypersensitive to cadmium. The cytotoxic impact of cadmium may be related to the cellular level of metallothioneins, which is frequently deregulated in cancer cells (62).

It would be naive to suggest that effects on p53 alone can explain all of the cadmium carcinogenicity. Indeed, it is likely that cadmium substitutes for zinc and alters the function of a number of other cellular proteins. For example, Cd2+ (as well as a number of other metal ions) has been shown to alter the nucleotide selectivity of human DNA polymerase β in vitro (63). In addition, factors such as competition between metals and interactions with metallothioneins should also be considered. However, we believe that our observations represent a important step in the understanding of the carcinogenic potential of cadmium. Moreover, these observations also provide a model system for determining how essential metals such as zinc or metal chelators may be used in preventive approaches to reduce cadmium carcinogenesis.

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