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Pyrrolidine Dithiocarbamate Prevents p53 Activation and Promotes p53 Cysteine Residue Oxidation*

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Pyrrolidine dithiocarbamate (PDTC) is a thiol compound widely used to study the activation of redox-sensitive transcription factors. Although normally used as an antioxidant, PDTC has been shown to exert pro-oxidant activity on proteins both *in vitro* and *in vivo*. Because p53 redox status has been shown to alter its DNA binding capability, we decided to test the effect of PDTC on p53 activation. In this communication, we report that PDTC inhibits the activation of temperature-sensitive murine p53[Val-135] (TSp53) in the transformed rat embryo fibroblast line, A1-5, as well as wild-type human p53 in the normal diploid fibroblast line, WS1neo. In A1-5 cells, PDTC abrogated UV- and temperature shift-induced TSp53 nuclear translocation and p53-mediated transactivation of *MDM2*. PDTC also blocked UV-induced accumulation of wild-type p53 in WS1neo cells. Continual presence of PDTC was required for its effect as both UV-induced nuclear translocation and accumulation resumed after PDTC removal. We next investigated whether PDTC treatment altered the p53 redox state. We found that PDTC increased p53 cysteine residue oxidation *in vivo*. This represents the first direct evidence showing that the p53 redox state can be altered *in vivo* and that increased oxidation correlates with its inability to perform its downstream functions.

The p53 tumor suppressor protein is believed to play an important role in maintaining genomic integrity and preventing tumorigenesis. A high frequency of gene-inactivating mutations observed in a wide variety of human cancers demonstrates the importance of functional inactivation of p53 in cell malignancy (1). Part of the mechanism of its function is based on its transcription regulation of some crucial genes, such as *WAF1/CIP1/SDI1*, a cyclin kinase inhibitor that leads to cell growth suppression (2, 3) and *MDM2*, a p53 feedback inhibition gene (4, 5). Nuclear localization of p53 appears to be essential to mediate downstream events (6). Nuclear accumulation of p53 is mediated by three specific nuclear localization signals inherent in the primary structure of the protein, which encompass residues 310–319, 369–375, and 379–384 (6). Mutations in the first nuclear localization signal (residues 310–319) hinder its nuclear translocation and result in inactivation of its transformation suppressor function (7, 8). However, some mechanisms of p53 inactivation appear to prevent the ability of p53 to reside in the nucleus without mutating the *p53* gene. For example, in some inflammatory breast cancers, undifferenti-

ated neuroblastomas and retinoblastoma cells expressing wild-type p53, the protein appears to be partially inactivated by cytoplasmic sequestration (9–11). Furthermore, the high tumorigenesis rate in the livers of transgenic mice expressing the hepatitis B viral HBx protein is probably linked to the sequestration and functional inactivation of p53 in the cytoplasm by the HBx protein (12).

Other types of p53 inactivation have been reported in cancers. A well-characterized example is the degradation of p53 by human papillomavirus (HPV)[1] E6 protein in cervical cancer. The E6 oncoprotein, expressed by oncogenic subtypes of HPV, binds p53 and directs its destruction through a ubiquitin-mediated pathway (13). Furthermore, a defective p53 response to ionizing radiation is observed in cells lacking the *ATM* gene, the gene mutated in ataxia-telangiectasia patients (14). After γ-radiation, p53 in such cells is not correctly induced, thus, impairing its G_1 arrest function (14, 15). Finally, the MDM2 cellular oncoprotein appears to be required to regulate p53 levels and its transactivation activity. Abnormal overexpression of *MDM2* can lead to p53 inhibition in a variety of cancers (16, 17).

Various forms of stress, such as ionizing radiation, UV radiation, medium depletion, hypoxia, heat shock, ribonucleotide depletion, and calcium phosphate treatment, lead to the induction of p53 protein level and the accumulation of transcriptionally active p53 inside the nucleus (18–24). Several other transactivators, such as NF-κB, AP-1, and Egr-1, can also be activated by UV radiation and other types of stressors (25–27). How stressors stimulate cellular responses is not completely known. One hypothesis is that reactive oxygen intermediates (ROIs), commonly produced by many of these stressors, act as second messengers for the activation of these transactivators. Paradoxically, the DNA binding activity of some of these transactivators, including p53, is dependent on maintaining a low redox potential of these proteins (28–30). In order to properly control the activation of these important transactivators, it is possible that the redox state of these proteins is highly regulated inside the cell.

In this study, we investigated p53 activation in intact cells by analyzing the effects of pyrrolidine dithiocarbamate (PDTC), a widely used compound in redox regulation studies of NF-κB and AP-1 (25, 31–32). PDTC contains two thiol moieties that can chelate metal ions and may exert either antioxidant or pro-oxidant effects (33). Here we demonstrate that PDTC inhibits p53 nuclear translocation and p53 induction. The inhib-

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[1] The abbreviations used are: HPV, human papillomavirus; DHR, dihydrorhodamine 123; DTT, dithiothreitol; FACS, fluorescence activated cell sorter; IF, indirect immunofluorescence; MPB, 3-(maleimidopropioryl)biocytin; NEM, *N*-ethylmaleimide; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediate; PAGE, polyacrylamide gel electrophoresis.

itory action of PDTC is not mediated by scavenging peroxides, but rather through alteration of the p53 redox state.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The A1-5 rat embryo fibroblast cell line was maintained and grown in 90% Dulbecco's modified Eagle's medium supplemented with 4500 mg/liter glucose and 2 mM L-glutamine (Irvine Scientific, Irvine, CA), 10% heat-inactivated fetal bovine serum (Gemini Bioproducts), and penicillin (100 units/ml)-streptomycin (100 mg/ml) solution (Irvine Scientific) with 5% CO_2 at 37 °C. WS1neo and WS1E6 cell lines were kind gifts from Drs. Geoffrey Wahl and Steven Linke at the Salk Institute, La Jolla, CA. Cells were maintained at 37 °C in modified Eagle's medium (Irvine Scientific) with 1× nonessential amino acids (Irvine Scientific), 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 200 μg/ml G418 (Life Technologies, Inc.). PDTC and *N*-ethylmaleimide (NEM) were purchased from Sigma. Dihydrorhodamine 123 (DHR), 3-(maleimidopropioryl)biocytin (MPB) and NutrAvidin were purchased from Molecular Probes (Eugene, OR). Dithiothreitol (DTT) was purchased from Fisher Biotech (Fair Lawn, NJ). Slide chambers were from Nunc, Inc. (Naperville, IL). All antibodies used in this study (except PAb421) were purchased from Oncogene Research Products (Cambridge, MA).

UV Treatment—A1-5 cells were seeded in 10-cm plates (5×10^5 cells) or 2-well slide chambers (3×10^4 cells) and grown at 37 °C overnight followed by incubation at 39 °C for another 24 h. Medium was removed, and cells were exposed to UV radiation from a germicidal lamp (254 nm) at 1.7–1.9 $J/m^2/sec$ monitored by a radiometer (UVP Inc., Upland, CA). After UV treatment, prewarmed fresh medium was applied to the cultures, and they were returned to the incubator. For human diploid fibroblast lines, a 1:3 dilution of WS1neo and 1:5 dilution of WS1E6 from a confluent 10-cm plate were used to seed the plates 1 day prior to the treatment.

Indirect Immunofluorescence (IF) Staining—IF staining was carried out as described previously (34). The intensity of fluorescence in the cytoplasm and nucleus was quantified from film negatives using IPLab Gel software (Signal Analytics Corp., Vienna, VA). For each densitometric value, 20 cells were counted, and the S.D. was calculated using Microsoft Excel software (version 2.0). Percentage of nuclear intensity was calculated by dividing the nuclear fluorescence level by the nuclear plus the cytoplasmic fluorescence level.

Temperature Shift Experiment—A1-5 cells were grown at 37 °C overnight followed by incubation at 39 °C (or 32 °C as control) for another 24 h. Prewarmed (32 °C) medium with or without PDTC was then supplied to the cells before switching to 32 °C for further incubation at time periods indicated in the figure legends.

Cell Harvesting and Western Blotting—At each indicated time point, cells were washed once with 5 ml of PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) and harvested in 2 ml of cold PBS with a cell scraper. Cell pellets were obtained by centrifugation at 1600 rpm in a tabletop centrifuge (Beckman model T J-6) for 5 min and stored at −80 °C. Pellets from 10-cm plates were ruptured by sonication in 1 ml (if A1-5 cells were used) or 100 μl (if WS1neo or WS1E6 were used) of lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 150 mM sodium chloride, 0.5% Nonidet P-40) freshly supplied with proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM E64, 1 μM leupeptin, and 1 μM aprotinin). Soluble protein concentration was determined by Bio-Rad protein assay (Bio-Rad), and 40 μg (or other amount as indicated) of total protein was resolved on a 10 or 12% SDS-PAGE gel. Protein transfer to Immobilon-P membrane (Millipore Co., Bedford, MA) and Western blotting were performed as described previously (35). Purified PAb421 hybridoma supernatant (34) or DO-1 was used as the primary antibody to detect p53 from A1-5 cells or human fibroblasts respectively. IPLab Gel software was used to quantify band intensities.

FACS Analysis of Peroxide Levels—Levels of peroxides were determined by FACS analysis as described elsewhere (26). Briefly, A1-5 cells, growing at 39 °C, were preincubated with 4 μM DHR for 30 min followed by 50 J/m^2 UV radiation or changing of the incubation temperature to 32 °C. After UV radiation or temperature shift, DHR incubation was continued for 30 min more in the presence or absence of PDTC. The intensity of fluorescence of rhodamine 123 (wavelength 500–540 nm), which was converted intracellularly from DHR, was assessed from 50,000 cells by flow cytometry with an excitation source of 488 nm.

Selective Labeling of Oxidized Cysteine Residues—This procedure is a modified form of the method described by Bayer *et al.* (36). Cells (1.4×10^6) were seeded into 15-cm plates. For each condition, two 15-cm plates were used. Frozen cell pellets were lysed by sonication in 1 ml of SEE

(0.1 M sodium phosphate, pH 7.0, 5 mM EDTA, and 5 mM EGTA) with 20 mM NEM and 1 mM phenylmethylsulfonyl fluoride. After centrifugation to remove insoluble material and subsequent protein concentration measurements, soluble lysates were diluted to 0.6 mg/ml with SEE plus NEM and phenylmethylsulfonyl fluoride. After incubation on wet ice for 30 min, diluted lysates were individually dialyzed against SEE overnight with one change of buffer after the first 4 h. To reduce oxidized sulfhydryl groups, DTT was added to 1.5 ml of sample to a concentration of 20 mM. After 30 min of incubation on wet ice, the samples were individually dialyzed as described above. MPB (10 μg/ml) was added to DTT-treated or DTT mock-treated samples at 4 °C for 30 min followed by dialysis. Samples were again measured for protein concentration. Immunoprecipitation of 50 μg of each sample with a p53-specific antibody mixture (1.8 μg of PAb421 and 0.6 μg of PAb240) or 2.4 μg of anti-E1A antibody (negative control) was performed as described previously (34). The immunoprecipitated proteins were analyzed by 8% SDS-PAGE. Duplicate immunoprecipitations of each sample and SDS-PAGE were performed to determine the immunoprecipitation efficiency. After electroblotting to Immobilon-P membranes, one membrane was probed with NutrAvidin (avidin conjugated with horseradish peroxidase) to detect proteins modified by MPB, and the other membrane was probed with PAb421 to determine the amount of p53 in each sample. To ensure that MPB modified proteins with disulfide linkages and not proteins with free sulfhydryl groups, two purified proteins were used as controls. One was bovine pancreas chymotrypsinogen (Worthington, Freehold, NJ), which has five disulfide linkages (37). The second was rabbit muscle aldolase (Worthington), which has eight free sulfhydryl groups/subunit but no disulfide linkages (38).

RESULTS

PDTC Inhibits p53 Nuclear Translocation and Transactivation—To study the mechanism of p53 activation, we used a transformed rat embryo fibroblast cell line, A1-5 (39), which expresses a high level of a temperature-sensitive mutant p53, TSp53. TSp53 is a protein that expresses a valine residue at codon 135 instead of alanine. This TSp53 is located in the cytoplasm at the nonpermissive temperature, 39 °C, but in the nucleus when cells are incubated at 32 °C (Fig. 1, *far left panels*) (39, 40). To demonstrate that the normal upstream p53 signaling pathway in this cell line is intact, we tested whether TSp53 was capable of translocating into the nucleus in response to UV radiation at the nonpermissive temperature. The ability of TSp53 to accumulate in the nucleus in response to UV radiation was tested by treating cells with 50 J/m^2 of UVC light. As shown in Fig. 1 (*top row*), at 2 h post-irradiation, p53 started to appear in the nucleus, and by 6 h post-irradiation, 60% of the total cellular p53 was detected in the nucleus as compared with 29% prior to radiation (Figs. 1 and 2*B*). A time course study was also conducted to determine p53 nuclear accumulation after temperature shift from 39 to 32 °C (Fig. 1, *bottom right three panels*). After 2 h, p53 nuclear accumulation was apparent, and by 6 h, p53 nuclear accumulation was complete, with very little cytoplasmic p53 remaining. Although no change in p53 steady state level was observed after UV radiation or temperature shift (data not shown), the ability of TSp53 to respond to UV at the nonpermissive temperature suggests that the upstream pathway for p53 activation in response to DNA damage is intact in this cell line.

The effect of the thiol-containing agent PDTC on p53 activation was then investigated. When PDTC was applied to A1-5 cells immediately after UV treatment, TSp53 nuclear translocation was completely abrogated (Fig. 2*A*). In the presence of PDTC, the proportion of cellular p53 residing inside the nucleus (25 and 30% at 2 and 4 h postirradiation, respectively) after UV treatment was similar to that of non-UV treated cells (29%) (Fig. 2*B*). Interestingly, PDTC also prevented TSp53 nuclear translocation induced by temperature shift. No increase of nuclear p53 was observed if PDTC was present during temperature shift from 39 to 32 °C, whereas a 4-fold increase was observed in the absence of PDTC (Fig. 2, *C* and *D*). The inhibition of TSp53 nuclear translocation by PDTC during tem-

FIG. 1. **UV radiation and temperature shift from 39 to 32 °C trigger p53 nuclear translocation in A1-5 cells.** A1-5 cells were grown at 39 °C and treated with 50 J/m² UV light followed by further incubation at 39 °C (*top row*). Cell incubation temperature was shifted from 39 to 32 °C for 2, 4, and 6 h (*bottom row, three right panels*). Cells were incubated at 32 °C for 24 h (*bottom left panel*). At indicated time points, cells were fixed with acetone and stained with PAb421 antibody against p53 followed by FITC-conjugated secondary antibody (magnification, × 100).
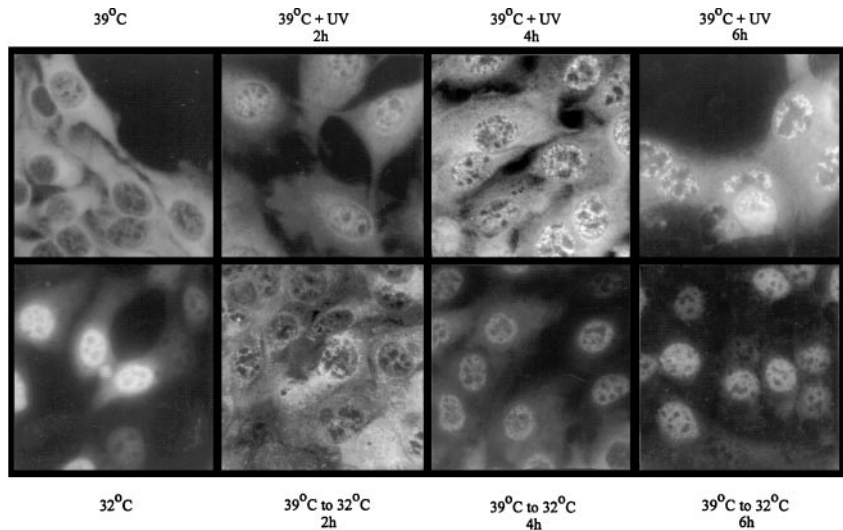
perature shift was dose-dependent. The presence of 5 μM PDTC had no effect on TSp53 nuclear translocation, whereas 10 μM showed moderate inhibition. Only upon application of 20 μM or more PDTC was complete inhibition of translocation observed (data not shown). In sum, we observe the prevention of both UV- and temperature shift-mediated TSp53 nuclear translocation by PDTC.

In order to confirm that p53 activity was inhibited, the expression of a p53 downstream effector, *MDM2*, was examined. To do this, we shifted the incubation temperature of A1-5 cells from 39 to 32 °C to induce *MDM2* expression (4, 5). In Fig. 3 we show that p53 normally activates MDM2 protein expression within 2 h after shifting the incubation temperature (Fig. 3, *lanes 4* and *5*). However, in the presence of PDTC, temperature shift failed to induce *MDM2* expression (Fig. 3, *lanes 8* and *9*). This suggests that by excluding p53 from the nucleus, PDTC is able to prevent p53-mediated transactivation of *MDM2*. It should be noted that we found no induction of MDM2 after UV treatment. The lack of MDM2 induction by the high dose of UV may be due to transcriptional repression of *MDM2* (41, 42) or because p53 fails to adopt a transcriptionally competent state inside the nucleus at the nonpermissive temperature.

To further characterize the inhibitory effect, we tested whether the continual presence of PDTC was required to prevent temperature shift-induced p53 nuclear translocation. PDTC was added to cells immediately after temperature shift to prevent p53 translocation but removed after 2 h. The cells were then maintained for another 6 h at the permissive temperature with fresh medium. When both the p53 localization and the expression of *MDM2* were examined, we found that the removal of PDTC led to an increase in nuclear p53 level (Fig. 2*D, last column*) and *MDM2* expression (Fig. 3, *lane 10*). This demonstrates that p53 activity recovers once PDTC is removed. We then tested whether PDTC was continually required to prevent p53 nuclear translocation after UV treatment. Cells were pretreated with PDTC for 30 min prior to UV radiation. After UV treatment, fresh medium was added without PDTC. As shown in Fig. 4 (*columns 4* and *5*) p53 accumulated into the nucleus within 4 h. All of the data demonstrate that PDTC prevents p53 translocation and its transactivation function and that PDTC must be continually present for its effect.

PDTC Inhibits UV-induced p53 Level Increase and HPV E6-dependent p53 Degradation in Normal Human Fibroblasts— Because all previous studies were conducted with TSp53, it was important to determine whether PDTC could also inhibit wild-type p53 activity in normal human diploid cells. The human fibroblast cell line WS1neo, expressing a retrovirally inserted

neomycin resistance gene, was used for this study (24). Previous work demonstrated that ionizing radiation and nucleotide depletion induce p53 expression and cell cycle arrest in these cells. As shown in Fig. 5, within 4 h after UV treatment, p53 levels increased 140% (2.4-fold). When PDTC was added to WS1neo cells, the UV-induced increase in p53 level was almost completely inhibited after 4 h (Fig. 5, *lane 4 versus lane 5*). In the presence of PDTC, p53 increased only 30% (1.3-fold) after UV treatment. Similar to A1-5 cells, inhibition by PDTC was reversible. Subsequent removal of PDTC after 4 h, followed by continual incubation in fresh medium, restored the p53 UV response. Thus, in UV-treated cells, the p53 level at 20 h after PDTC removal was identical to the p53 level in cells not treated with PDTC (Fig. 5, *lanes 8* and *9*). This demonstrates that, as in A1-5 cells, PDTC was able to temporarily inhibit the ability of p53 to respond to UV radiation. Curiously, after 24 h, we observed an intermediate p53 increase of approximately 90–150% in cells that were not exposed to UV light, both in the presence and the absence of a 4-h PDTC treatment (Fig. 5, *lanes 6* and *7*). The cause of this intermediate p53 induction may be due to the mock treatment (medium removal and replenishment). However, the fact that the level of p53 induction was almost identical in these two samples indicates that PDTC alone had little effect on p53 induction. This experiment also shows that the UV-induced signal is maintained for at least 4 h during PDTC treatment, although p53 is not able to respond within this period due to the presence of PDTC. Whether this means that the damage elicited by UV is maintained throughout this period or a UV-mediated signal is stable throughout this period is unclear. Our data indicate that p53 is able to respond to UV up to 4 h post-irradiation.

Surprisingly, we also observed an inhibition of HPV E6-mediated degradation of p53 by PDTC. Human fibroblasts expressing E6 (WS1E6) have no detectable p53 because E6 mediates rapid p53 degradation (43, 44). As expected, no p53 was present before or after UV radiation in cells expressing E6 (Fig. 5, *lanes 10* and *12*). Nevertheless, p53 was observed in WS1E6 during PDTC treatment independent of UV radiation (Fig. 5, *lanes 11* and *13*), and p53 degradation resumed within 10 h after the removal of PDTC (data not shown). The ability of PDTC to prevent p53 nuclear translocation, p53 induction, p53-mediated transactivation, and E6-mediated p53 rapid degradation suggests that PDTC may be able to directly interfere with p53 protein itself.

Temperature Shift Does Not Induce Peroxide Formation— ROIs such as superoxide anion ($O_2^{\bar{}}$), peroxides (ROOR), or hydroxyl radicals (OH·) are believed to act as secondary mes-

A

without PDTC   **with PDTC**

a   c

2h

b   d

4h

B

C

mock treated   **with PDTC**

a   c
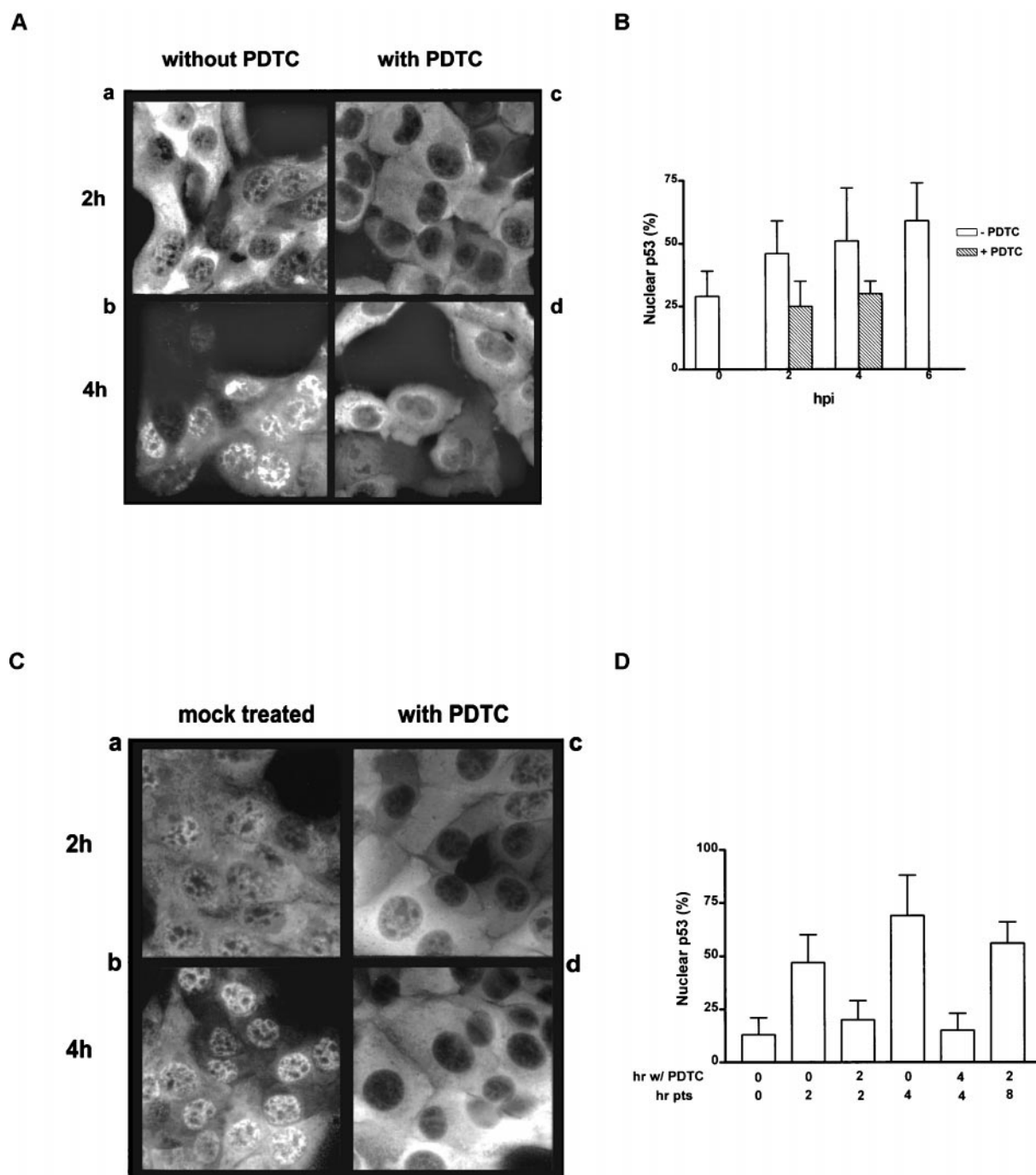
2h

b   d

4h

D

FIG. 2. **p53 nuclear translocation was suppressed in the presence of PDTC.** *A* and *B*, at 39 °C, A1-5 cells were treated with 50 J/m² UV radiation followed by further incubation at 39 °C in the presence or absence of 25 μM PDTC. A, IF analysis at 2 (*a*) and 4 (*b*) h after UV radiation; IF analysis at 2 (*c*) and 4 (*d*) h after UV radiation in the presence of 25 μM of PDTC. *B*, percentage of nuclear p53 in the presence or absence of PDTC after UV radiation. (hpi, hours of incubation post-irradiation). *Stippled bar*, in the presence of 25 μM PDTC after UV radiation; *clear bar*, mock PDTC-treatment after UV radiation. *C* and *D*, A1-5 cells were first incubated at 39 °C overnight. Cells were then moved to the 32 °C incubator after replacing the medium with fresh medium or medium containing 25 μM PDTC. *C*, the subcellular localization of p53 was detected by IF. IF analysis at 2 (*a*) and 4 (*b*) h after temperature shift to 32 °C; IF analysis at 2 (*c*) and 4 (*d*) h after temperature shift to 32 °C in the presence of 25 μM of PDTC. *D*, the percentage of nuclear p53 in the presence or absence of 25 μM PDTC during temperature shift from 39 to 32 °C. Cells were incubated in the presence or in the absence of PDTC for the indicated periods after temperature shift. After incubation with PDTC, cells were either immediately fixed or washed and allowed to incubate further at 32 °C (*hrs pts*, hours of incubation post-temperature shift).

sengers in the signal transduction pathway of several transactivators. Because hydrogen peroxide alone was shown to induce nuclear accumulation of p53 (Refs. 23 and 45 and data not shown), it was possible that PDTC inhibits p53 activation through scavenging peroxides generated during UV treatment or temperature shift. If this is the case, a change of intracellular peroxide level should be found in all situations in which PDTC inhibits p53 activation. The intracellular peroxide level

after UV treatment or temperature shift was then determined by the ability of the membrane permeable nonfluorescent substrate, DHR, to react with peroxide within the cell and oxidize to rhodamine 123, a membrane impermeable fluorescent product (46, 47). A1-5 cells were preincubated at 39 °C for 30 min with DHR, UV treated or temperature shifted, and then harvested after another 30 min. As expected, the intracellular level of peroxides increased 50–100% after UV radiation (Fig. 6).
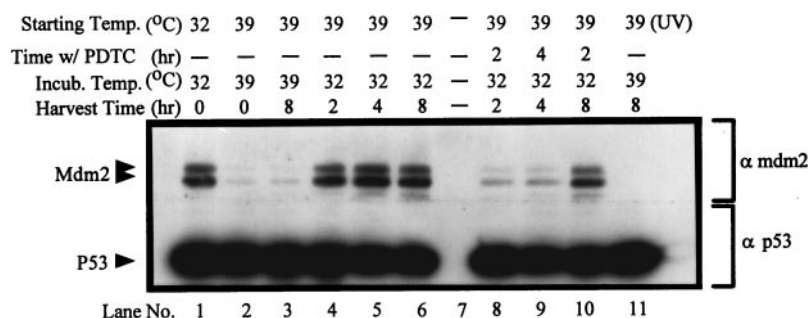
Starting Temp. (°C)	32	39	39	39	39	39	—	39	39	39	39 (UV)
Time w/ PDTC (hr)	—	—	—	—	—	—	—	2	4	2	—
Incub. Temp. (°C)	32	39	39	32	32	32	—	32	32	32	39
Harvest Time (hr)	0	0	8	2	4	8	—	2	4	8	8

Mdm2 ▶▶ α mdm2

P53 ▶ α p53

Lane No.   1   2   3   4   5   6   7   8   9   10   11

FIG. 3. **Continual presence of PDTC is required for its inhibitory effect on p53-mediated expression of *MDM2*.** A1-5 whole cell soluble lysate (40 μg) was separated by 12% SDS-PAGE and transferred to Immobilon-P membrane. The membrane was cut horizontally at the 68-kDa marker. The lower portion was probed with PAb421 for p53 detection (*α p53*), and the upper portion was probed with 2A10 antibody against MDM2 (*α mdm-2*). *Lane 1*, cells grown at 32 °C overnight; *lane 2*, cells grown at 39 °C overnight; *lane 3*, cells grown at 39 °C overnight, mock-UV treated, and further incubated for another 8 h; *lanes 4–6*, the incubation temperature of the cells was shifted from 39 to 32 °C for 2, 4, and 8 h, as indicated; *lane 7*, blank; *lanes 8* and *9*, cells were first incubated at 39 °C and then incubated at 32 °C in the presence of 25 μM PDTC for 2 and 4 h, respectively; *lane 10*, cells were first grown at 39 °C and then incubated at 32 °C in the presence of PDTC for 2 h. The medium containing PDTC was subsequently removed, and cells were washed once and supplied with fresh medium while they remained at 32 °C for another 6 h. *Lane 11*, cells were first grown at 39 °C and treated with 50 J/m² UV, after which cells were further incubated at 39 °C for 8 h.
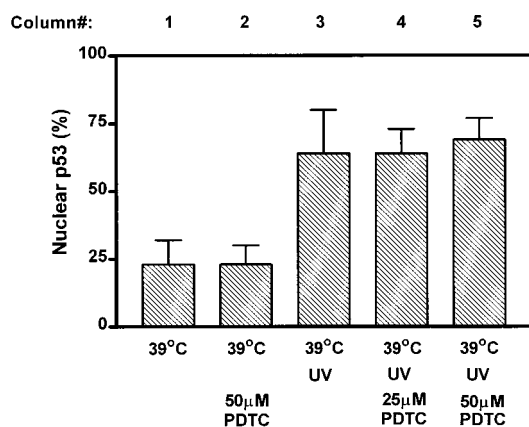

FIG. 4. **Inhibition of UV-induced p53 nuclear translocation requires continual presence of PDTC.** At 39 °C, A1-5 cells were treated with 25 or 50 μM PDTC for 30 min. Cells were then UV treated, subsequently washed with fresh medium, and incubated for another 6 h at 39 °C with fresh medium without PDTC. After IF analysis, the percentage of nuclear p53 was calculated. *Column 1*, untreated cells; *column 2*, cells were treated only with medium containing PDTC but not UV; *column 3*, cells were UV-treated but not treated with PDTC; *columns 4* and *5*, cells were treated with 25 or 50 μM PDTC followed by UV treatment.

FIG. 5. **PDTC inhibited UV-induced accumulation and HPV E6-dependent degradation of wild-type p53 in normal human fibroblasts.** WS1neo or WS1E6 soluble whole cell lysate (40 μg) was separated by 10% SDS-PAGE and transferred to Immobilon-P membrane. The membrane was probed with antibodies to detect p53 as described under "Experimental Procedures." PDTC (10 μM) was supplied in the medium for 4 h (+). +, UV treatment (20 J/m²) prior to PDTC addition; −, cells mock treated with UV; hpi, hours of incubation post-irradiation. The relative level of p53 was quantified by comparing the p53 band intensity with that from cells harvested before any treatment (*lane 1*). In *lanes 1–9*, WS1neo cells were used, and in *lanes 10–13*, WS1E6 cells were used.

However, no alteration of peroxide level was observed during the temperature shift (Fig. 6). These observations show that PDTC inhibits p53 nuclear translocation independent of the peroxide level. We also found that PDTC, at p53 inhibitory concentration, only slightly inhibited UV-induced peroxide formation. The effect of PDTC on p53 appears to involve mechanisms distinct from the signal generated by peroxides.

PDTC Treatment Correlates with the Oxidation of Cysteine Residues on p53—PDTC has been shown to exert both pro- and antioxidant effects in cell-free and biological systems (33). Because PDTC appears to inhibit p53 activity in a peroxide-independent manner, we tested whether PDTC could alter the p53 oxidation state *in vivo*. Specifically, we checked whether p53 cysteine residues could undergo an S-thiolation reaction, defined as any disulfide bond formation (48). Such bonds could be formed intermolecularly or intramolecularly. Fig. 7 shows the experimental design for the detection of cysteine oxidation on p53. Endogenous protein free sulfhydryl groups were blocked by lysing the cells in the presence of NEM, a reagent that forms nonreducible thioether bonds with free sulfhydryl groups. After NEM treatment, proteins were treated with DTT to free disulfide-linked cysteine residues, and newly formed sulfhydryl groups were covalently modified with MPB, a biotin-
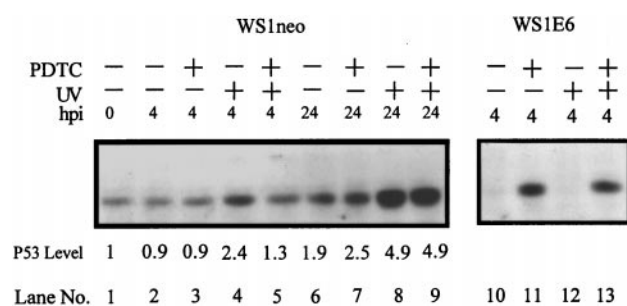
conjugated maleimide that, like NEM, forms a nonreducible thioether bond. MPB-modified p53 was then immunoprecipitated with an antibody mixture (PAb421 and PAb240) followed by SDS-PAGE analysis. After electroblotting onto polyvinylidene difluoride membrane, MPB-modified p53 was then detected with peroxidase-conjugated avidin followed by ECL. Immunoprecipitation efficiency was assessed by duplicate immunoprecipitations followed by standard p53 Western analysis.

A1-5 cells at 39 °C were treated with UV light. PDTC was immediately added, and the cells were incubated for 1.5 h prior to harvesting. As shown in Fig. 8*A*, cysteine residues on p53 were oxidized in the presence of PDTC (compare *lanes 1* and *3* with *lane 5*). The upper blot (Fig. 8*A, a*) is a chemiluminescence image showing the level of p53 that undergoes thiol modification. *Lanes 1, 3*, and *5* show that p53 and several p53-associated proteins are modified by MPB. *Lanes 2, 4*, and *6* show that MPB protein modification in *lanes 1, 3*, and *5* requires DTT treatment to expose sulfhydryl groups. UV treatment did not significantly alter the level of p53 S-thiolation (compare *lane 1* and *lane 3*). However, PDTC treatment consistently showed a 2–3-fold increase in p53 cysteine residue S-thiolation. We did not detect an increase in S-thiolation on p53-associated proteins (denoted with *asterisks*). Immunoprecipitation efficiency did not vary by more than 20% in any lane (Fig. 8*A, b*). We also

tested whether PDTC increased p53 cysteine S-thiolation in A1-5 cells incubated at 32 °C. In this experiment cells, growing at 39 °C were shifted to 32 °C for 1.5 h in the presence of PDTC. We found that p53 cysteine S-thiolation was increased only with PDTC treatment and not with temperature shift alone (Fig. 8*B*). The data show that PDTC treatment promotes p53 S-thiolation. Therefore, it is possible that direct modification of

p53 cysteine residues may lead to abrogation of p53 nuclear translocation, p53 level-increase, HPV-E6 mediated degradation of p53 and *MDM2* transactivation.

Interestingly, we also observed endogenous cysteine S-thiolation in the absence of any treatment (Fig. 8, *A* and *B, lanes 1* and *3*). The nature and the biological function of this cysteine modification are not known at this point.
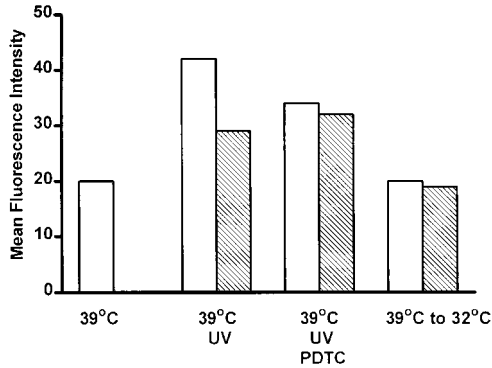

FIG. 6. **Temperature shift does not lead to a detectable increase in peroxide level.** For determination of the peroxide level generated after UV radiation, at 39 °C, A1-5 cells were preincubated with 4 μM DHR for 30 min followed by irradiation with 50 J/m^2 UV radiation and continual incubation at 39 °C in the presence or absence of 25 μM PDTC for 30 min. For determination of peroxide generation during temperature shift, A1-5 cells were preincubated with 4 μM DHR for 30 min at 39 °C. Cells were then placed in a 32 °C incubator for 30 min and harvested. For measurement of basal level of peroxide, cells were incubated at 39 °C with DHR for a total of 60 min. FACS analysis was used to measure the mean fluorescence intensity, which corresponds to the peroxide level inside the cell. *Hatched bar*, experiment 1; *clear bar*, experiment 2. Note that for experiment 2, a measurement of the basal peroxide level was not conducted.

DISCUSSION

In this study, PDTC was used to investigate redox regulation of p53. UV light induces p53 levels, p53 nuclear accumulation, and p53-mediated transactivation of several downstream effector genes, resulting in a delay in cell cycling (22, 49–50). PDTC has radical scavenging as well as pro-oxidant properties (25, 51). The evidence presented here shows that PDTC inhibits p53 induction, p53 nuclear translocation and p53-mediated transactivation of *MDM2*. Three principal findings of this study are: 1) PDTC inhibits p53 *in vivo*, 2) PDTC increases p53 cysteine residue oxidation, and 3) endogenous p53 contains oxidized cysteine residues.

PDTC Inhibits p53 Activation—The p53 protein exists in a latent state in cycling cells (52). Upon exposure to environmental stress, the p53 level increases and p53 accumulates in the nucleus (22, 49). Because superoxide anions (O_2^-), peroxides (ROOR), and perhaps hydroxyl radicals (OH·) are produced by UV treatment (53), the possibility that these ROIs are required for p53 nuclear accumulation was explored. We observed that the ROI scavenger PDTC inhibits p53 nuclear trafficking and prevents an increase in the steady state level of p53 after UV radiation. PDTC, however, does not inhibit p53 function by preventing peroxide generation. Evidence for this conclusion is based on the fact that PDTC blocked p53 nuclear translocation induced by temperature shift, a mechanism that does not pro-
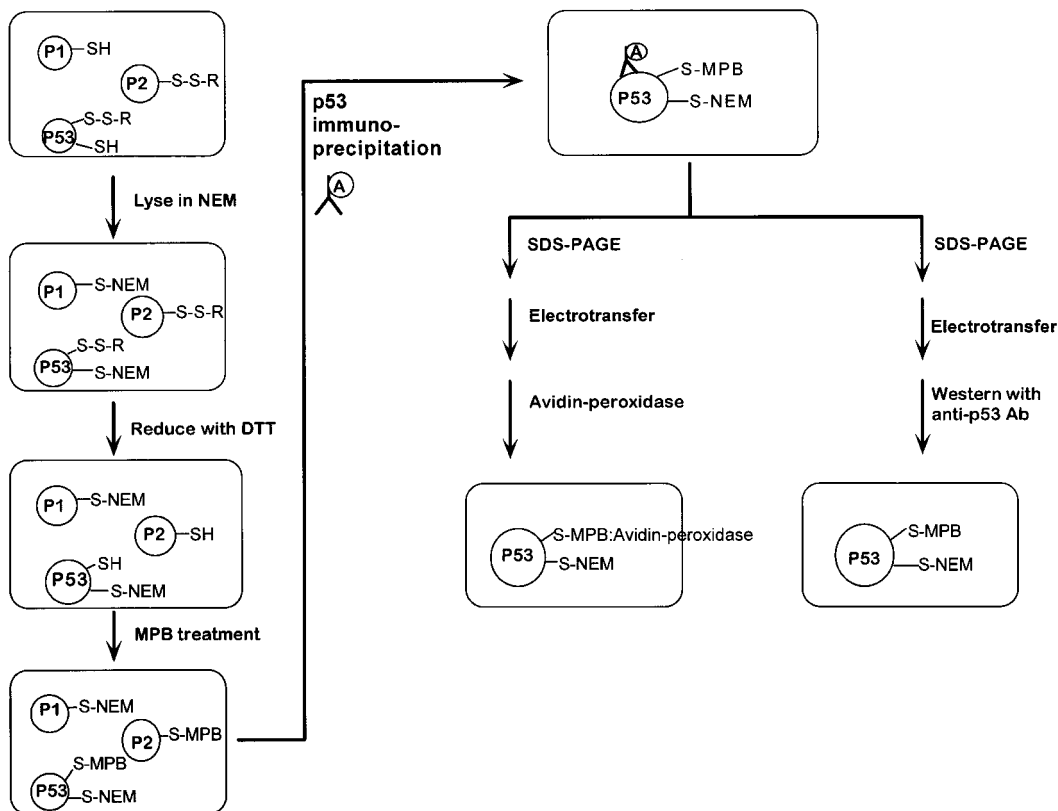

FIG. 7. **Experimental design to selectively detect p53 cysteine disulfide bonds.** *P1* and *P2* represent cellular proteins, other than p53, which contain free sulfhydryl groups (−*SH*) and disulfide bonds (−*S*−*S*−*R*), respectively. p53 protein is shown with two cysteine residues, one in a disulfide linkage and the other with a free sulfhydryl group. The *circled A* represents protein A-Sepharose. The *upside-down Y-shaped line* represents the p53-specific antibodies used for immunoprecipitation. See under "Experimental Procedures" for more details.
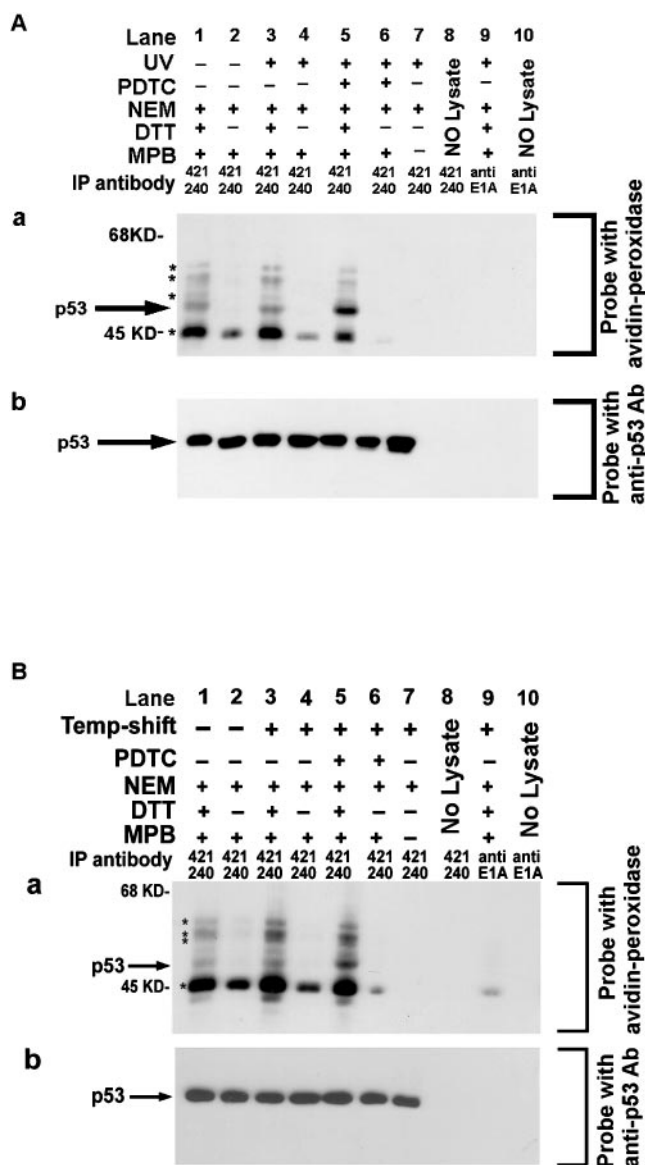
FIG. 8. **PDTC oxidizes p53 cysteine residues.** A1-5 cells were either UV-treated at 39 °C or incubated at 32 °C in the presence or absence of 25 μM PDTC. Cells were then harvested at 1.5 h post-treatment and lysed in the presence of 20 mM NEM. Oxidized cysteine residues on the proteins were selectively labeled with MPB followed by immunoprecipitation with p53-specific antibodies. *A*, soluble lysate from A1-5 cells mock-treated or UV-treated in the presence or absence of PDTC. *a*, probed with avidin-peroxidase to detect MPB labeled p53. *b*, Western blot to assess the p53 level after each treatment. *B*, soluble lysate from A1-5 cells incubated at 39 °C or temperature shifted to 32 °C in the presence or absence of PDTC. *a*, probed with avidin-peroxidase to detect MPB labeled p53. *b*, Western blot to assess the p53 level after each treatment. *Lanes 1* and *2* are soluble lysates from mock-treated cells; *lanes 3* and *4* are soluble lysates from UV-treated or temperature shifted cells; *lanes 5* and *6* are soluble lysates from UV-treated or temperature shifted cells in the presence of PDTC; *lane 7* is soluble lysate without MPB treatment; *lanes 8* and *10* are sham immunoprecipitations without cell lysate; *lane 9* is an immunoprecipitation of the soluble lysate used in *lane 3* with the non-p53 specific antibody anti-E1A.

duce detectable peroxides. Furthermore, because UV production of ROIs is very transient (53) ($t_{1/2}$ = 30 min) a 4-h PDTC treatment should be sufficient to permanently prevent p53 activation. Instead, we found that upon PDTC withdrawal 4 h after UV treatment, the p53 response resumed. The data are inconsistent with the radical scavenging property of PDTC. Similarly, another study found that hydrogen peroxide could

activate *WAF1/CIP1/SDI1*, another downstream effector gene of p53 (54). *WAF1/CIP1/SDI1* activation was delayed in the presence of PDTC but resumed after PDTC withdrawal. The data suggest that PDTC acts through a mechanism other than ROI scavenging to inhibit p53 translocation and up-regulation.

It was recently shown that the antibiotic geldanamycin can also prevent mutant p53 nuclear translocation (55). Geldanamycin probably inhibits p53 translocation by binding to hsp90 and interrupting the p53-hsp90 function. Geldanamycin also promotes rapid p53 destabilization (56, 57). PDTC does not act in the same manner as geldanamycin because it does not elicit p53 destabilization. Other studies have shown that p53 phosphorylation correlates with p53 induction after DNA damage (58, 59). One consequence of phosphorylation is inhibition of MDM2-p53 complex formation. MDM2-p53 complex formation leads to rapid p53 degradation (60, 61). PDTC, however, appears to act on p53 in a manner that is independent of MDM2 because it prevents p53 nuclear translocation in A1-5 cells at the nonpermissive temperature. At this temperature, little or no MDM2 is present.

PDTC Increases p53 Cysteine Residue S-thiolation—When the p53 redox state was examined, increased cysteine residue S-thiolation was observed during PDTC treatment. Because this modification was reversed by a reducing agent, it is likely that p53 cysteine residues undergo *S*-thiolation or thioesterification. This could explain why PDTC also prevented E6-mediated degradation of p53. If PDTC modifies a p53 cysteine residue critical for E6 binding, then p53 degradation would be blocked. In this regard, it previously was shown that a cysteine to tyrosine substitution at cysteine 135 of human p53 abolished binding between E6 and p53 and alleviated p53 degradation (62). Recently, p53 activation of *WAF1/CIP1/SDI1* and p53-DNA complex formation was shown to be blocked by PDTC via a mechanism that depends on Cu^{2+} (54). It was proposed that PDTC mediates a pro-oxidant effect by chelating Cu^{2+} and transporting it across the plasma membrane (51). Increased intracellular Cu^{2+} levels have been detected in cells treated with PDTC (51, 54).

How Cu^{2+} plus PDTC promotes p53 cysteine residue oxidation is not clear. Hainaut *et al.* (63) have shown that p53 directly binds Cu^{2+} *in vitro* (63). Thus, one mechanism could be that Cu^{2+} directs one-electron oxidation of the cysteine residue sulfhydryl bond, resulting in thiyl radical formation (64–66). This reactive radical could eventually lead to glutathione S-thiolation or intramolecular disulfide bond formation. If p53 cysteine residues critical for activation are modified by such a reaction, one might expect that reduction would increase p53 activities. In fact, DTT was observed to increase p53 DNA binding *in vitro* (29, 30). Ref-1, a protein that can regulate the redox state of a number of different proteins, was found to stimulate p53 DNA binding activity in a redox-dependent manner (67). In a recent study, a transgenic *Schizosaccharomyces pombe* strain that expresses transcriptionally active human p53 was created (68). Optimal transcriptional activation depended on the thioredoxin reductase gene, *TRR1*, the gene product of which has protein disulfide reductase activity. It appears that oxidation of p53 cysteine residues leads to inhibition of several of its activities.

Endogenous Cysteine Oxidation of p53—Interestingly, prior to PDTC treatment, we observe cysteine residue oxidation on p53 and on four or five uncharacterized p53 associated proteins. It is not clear whether such modifications are necessary for p53 activity. Cysteine to serine substitutions can model cases in which cysteines are in the reduced state. Cysteine to serine substitutions at conserved residues within the p53 central domain have delineated three sets of cysteine residues (69).

One set, at codons 173, 235, and 238 of mouse p53, is critical for optimal gene transactivation and cell transformation suppression activity. These residues directly interact with the zinc ion and therefore are probably necessary for structural integrity (70). A second set, at positions 121, 132, 138, and 272, is required for optimal gene transactivation and cell transformation but not DNA binding. A third set of cysteine residue substitutions, at positions 179 and 274, does not disturb any measured p53 activities. It is this second set of residues in which cysteine residue modification may actually be necessary for some p53 activity. Characterization of the molecule that modifies p53 cysteine residues and mapping of its site will give us more insight into the mechanism of p53 redox control.

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Pyrrolidine Dithiocarbamate Prevents p53 Activation and Promotes p53 Cysteine Residue Oxidation

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