p53 protein oxidation in cultured cells in response to pyrrolidine dithiocarbamate: a novel method for relating the amount of p53 oxidation *in vivo* to the regulation of p53-responsive genes

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A novel method was developed to determine the oxidation status of proteins in cultured cells. Methoxy-polyethylene glycol-maleimide MW 2000 (MAL-PEG) was used to covalently tag p53 protein that was oxidized at cysteine residues in cultured cells. Treatment of MCF7 breast cancer cells with pyrrolidine dithiocarbamate (PDTC), a metal chelator, resulted in a minimum of 25 % oxidation of p53. The oxidized p53 had an average of one cysteine residue oxidized per p53 protein molecule. The effect of PDTC treatment on downstream components of the p53 signal-transduction pathway was tested. PDTC treatment prevented actinomycin D-mediated up-regulation of two p53 effector gene products, murine double minute clone 2 oncoprotein and $p21^{WAF1/CIP1}$ (where WAF1 corresponds to wild-type p53-activated fragment 1 and CIP1 corresponds to cyclin-dependent kinase-interacting protein 1). Actinomycin D treatment led to accumulation of p53 protein in the nucleus. However, when cells were simultaneously treated with PDTC and actinomycin D, p53 accumulated in both the nucleus and the cytoplasm. The data indicate that an average of one cysteine residue per p53 protein molecule is highly sensitive to oxidation and that p53 can be efficiently oxidized by PDTC in cultured cells. PDTC-mediated oxidation of p53 correlates with altered p53 subcellular localization and reduced activation of p53 downstream effector genes. The novel method for detecting protein oxidation detailed in the present study may be used to determine the oxidation status of specific proteins in cells.

Key words: PDTC, methoxy-polyethylene glycol-maleimide, redox, tumour suppressor, nuclear accumulation.

INTRODUCTION

The proper response of the p53 tumour suppressor protein to certain oncoproteins and environmental stresses is believed to be an important step in the prevention of neoplastic transformation. Exposure of cells to these stresses typically results in p53 protein accumulation [1,2], binding of p53 to DNA in a sequence-specific fashion [3] and transactivation of a discrete set of genes [4–6]. Three types of post-translational modifications regulate p53 activity; phosphorylation, acetylation and oxidation [7]. Phosphorylation of p53 can prevent murine double minute clone 2 oncoprotein (MDM2)-mediated degradation of p53, ultimately leading to p53 protein accumulation [8,9]. Acetylation of p53 at lysine residues 320, 373 and 382 can increase p53 binding to DNA [10–12]. Unlike phosphorylation and acetylation, which constitute up-regulation signals for p53, oxidation of p53 appears to prevent p53 activation. Experimental treatment of p53 with oxidizing agents or thiol group-specific agents prevents p53 from binding to DNA [13–15].

Human p53 encodes ten cysteine residues all within the DNA binding domain of the protein. The thiol groups of these residues may be targets for oxidizing agents if these groups are accessible to oxidizing agents. X-ray crystallography structure analysis of the DNA binding domain of the p53 protein suggests that seven of these cysteine residues have exposed thiol groups on the surface of the protein [16]. In line with this analysis, recombinant p53 is highly susceptible to oxidation *in vitro* [13–15]. Rainwater et al. [15] showed that cysteine residues responsible for Zn^{2+} binding are also required for efficient DNA binding and for suppression of oncogene-mediated cell transformation.

While it is clear that p53 activity is sensitive to oxidizing agents *in vitro*, it was only recently demonstrated that the redox status of p53 could change in intact cells [17]. An increase in the level of oxidized p53 is observed when cultured cells overexpressing a temperature-sensitive p53 mutant are treated with a small metal-chelating agent, pyrrolidine dithiocarbamate (PDTC). Although the mechanism by which PDTC affects p53 oxidation is not clear, PDTC has been shown to increase the intracellular copper concentration [18–20]. This led to the suggestion that copper might mediate oxidation of exposed thiol groups on p53 [16]. Details of this mechanism await further clarification.

Reversible oxidation of protein thiol groups can take place *in vivo* by attachment of at least three different molecules to protein thiols. It has been known for some time that addition of glutathione to a protein produces an oxidized form that has been termed an S-glutathiolated protein [21]. Likewise, addition of a cysteine has been termed S-cysteylation [22]. More recently, there has been much interest in S-nitrosylated proteins that may be generated from nitric oxide or its related nitrosothiols [23]. Each

Abbreviations used: CIP1, cyclin-dependent kinase-interacting protein 1; CK, bovine heart muscle creatine kinase; DTT, dithiothreitol; MDM2, murine double minute clone 2 oncoprotein; MAL-PEG, methoxy-polyethylene glycol-maleimide MW 2000; NEM, *N*-ethylmaleimide; PDTC, pyrrolidine dithiocarbamate; Ref-1, redox factor-1; WAF1, wild-type p53-activated fragment 1.

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of these modifications is stable to isolation, but can easily be removed by simple reduction with a low molecular-mass thiol.

To further understand the mechanism that leads to p53 oxidation in cultured cells we developed a novel protein-thiol tagging procedure to study p53 oxidation directly. With this method we determined that PDTC treatment results in oxidation of at least 25% of wild-type p53 in MCF7 cells. Oxidized p53 in PDTC-treated cells had an average of one oxidation site per p53 protein molecule. PDTC treatment also led to inhibition of up-regulation of two p53 responsive genes and altered the nuclear accumulation properties of p53. The present study indicates that a significant proportion of p53 is susceptible to oxidation and that oxidation correlates with a loss of its activity in cultured cells.

EXPERIMENTAL

Cell culture

MCF7 cells were cultured in RPMI (Irvine Scientific, Santa Ana, CA, U.S.A.) containing 2 mM L-glutamine, 10% (v/v) heat inactivated fetal bovine serum (Irvine Scientific), 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate (Irvine Scientific) in humidified air/CO_2 (19:1) at 37 °C.

Antibodies and reagents

Anti-MDM2 antibody, SMP14, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-MDM2 antibody, 2A10, was obtained from hybridoma supernatant purified on a Protein A–Sepharose column. Anti-(p53 protein) antibodies (DO-1 and 1801) and anti-p21$^{WAF1/CIP1}$ (where WAF1 corresponds to wild-type p53-activated fragment 1 and CIP1 corresponds to cyclin-dependent kinase-interacting protein 1) antibody (Ab-1) were purchased from CalBiochem (San Diego, CA, U.S.A.). Actinomycin D, PDTC and bovine heart muscle creatine kinase (CK) were purchased from Sigma (St. Louis, MO, U.S.A.). Methoxy-polyethylene glycol-maleimide MW 2000 (MAL-PEG) was purchased from Shearwater Polymers, Inc. (Huntsville, AL, U.S.A.).

Tagging of CK

A novel method using MAL-PEG to tag oxidized sites on proteins was used. This method takes advantage of the fact that maleimide-containing molecules react with free thiol groups. This results in the formation of a covalent linkage between the maleimide and the protein. Two different maleimides were used, *N*-ethylmaleimide (NEM) and MAL-PEG. NEM, with a molecular mass of 125 Da, was used to block all of the free thiol groups exposed, and the second maleimide, MAL-PEG, with a molecular mass of 2000 Da, was used to tag disulphide-linked thiols after reduction by dithiothreitol (DTT). The MAL-PEG-tagged protein, with retarded mobility due to its covalent linkage with MAL-PEG, can be easily identified after separation by SDS/PAGE.

CK was oxidized by treatment with hydroxyethyl disulphide as previously described [24]. All further manipulations took place at 0–4 °C. S-thiolated CK, at a concentration of 3 mg/ml, was treated with 40 mM NEM for 1 h to block any sites that may have been reactive to maleimide. After exhaustive dialysis, CK was treated with 20 mM DTT to reduce mixed disulphide linkage. Isoelectric focusing analysis showed that DTT treatment completely removed the 2-hydroxyethanol adduct. CK was then treated for 1 h with 4 mM MAL-PEG to tag the free thiol group on the protein. The tagging reaction was quenched by treatment with excess 2-mercaptoethanol, a thiol-containing agent capable of reacting with the remaining MAL-PEG. Tagged CK was analysed by SDS/PAGE (10% polyacrylamide) and visualized by staining with 0.2% Coomassie Blue-R (Sigma). Differences in the mobility of the tagged protein and the untagged protein serves as an indicator for identification of oxidized protein.

Treatment of cultured cells with PDTC

Four confluent 15-cm plates of MCF7 cells were treated with either 60 μM PDTC or 120 μM PDTC in 15 ml of fresh medium for 5 h. Vehicle (control)-treated plates were treated with medium supplemented with PBS solution. Cells were harvested by scraping with a rubber policeman. The suspension was centrifuged for 10 min at 1000 ***g*** and the supernatant was removed. Cell pellets were stored at −80 °C. Each cell pellet (from four plates) was thawed and sonicated at 0 °C in 2 ml of SEEN [0.1 M sodium phosphate (pH 7.0), 5 mM EDTA, 5 mM EGTA and 0.1% Nonidet P40] supplemented with 20 mM NEM and 1 mM PMSF. The crude cell lysate mixture was centrifuged to remove particulate matter and the soluble protein concentration was measured and adjusted to 0.6 mg/ml with SEEN/NEM/PMSF solution, and maintained at 0 °C for an additional 1 h with occasional mixing. Each sample was dialysed against SEEN to remove unreacted NEM. After dialysis, one-half of each sample was stored at 4 °C until the MAL-PEG tagging step. This sample served as a control for specificity of MAL-PEG tagging. The second half was mixed with 20 mM DTT and incubated for 1 h at 0 °C. The DTT-treated samples were dialysed, and MAL-PEG was added to both samples (with and without DTT treatment) at a final concentration of 300 μM and subsequently incubated for 1 h at 0 °C. Protein concentrations of treated samples were determined by the Bradford assay and samples were separated by SDS/PAGE (7.5% or 10% polyacrylamide). Electroblotting and Western-blot analysis were performed as previously described [17]. The relative levels of oxidized and non-oxidized p53 were obtained from densitometry analysis of scanned autoradiographs using UN-SCAN-IT gel software (Version 5.1). Antibodies were used according to the manufacturer's instructions.

Immunofluorescence studies

Indirect immunofluorescence was performed as previously described [25]. In brief, 2×10^4 cells/well were plated on slide chambers (Nalge Nunc International, Rochester, NY, U.S.A.) 24 h prior to treatment. Cells were treated with 16 nM actinomycin D and 60 μM PDTC, and incubated for 5 h at 37 °C. Cells were fixed in acetone/methanol (1:1, v/v) for 2 min at −20 °C. Cells were stained for p53 with anti-p53 monoclonal antibody 1801 at a final concentration of 2 μg/ml and incubated overnight at 4 °C. For fluorescence detection, cells were treated with FITC-conjugated sheep F(ab)$_2$ fragment anti-mouse IgG (Sigma) at 1:100 dilution for 2 h at 37 °C. To photograph a given field of cells the aperture of the camera was held open for 1 min.

RESULTS

Tagging oxidized protein cysteine residues

A thiol-group tagging procedure was developed to measure protein oxidation in cultured cells. A diagram outlining this procedure is presented in Figure 1(A). First, cells were lysed in the presence of excess NEM to block all reactive thiol groups (Step 1). The crude cell lysate was then centrifuged to remove particulate matter and the soluble lysate was dialysed to remove

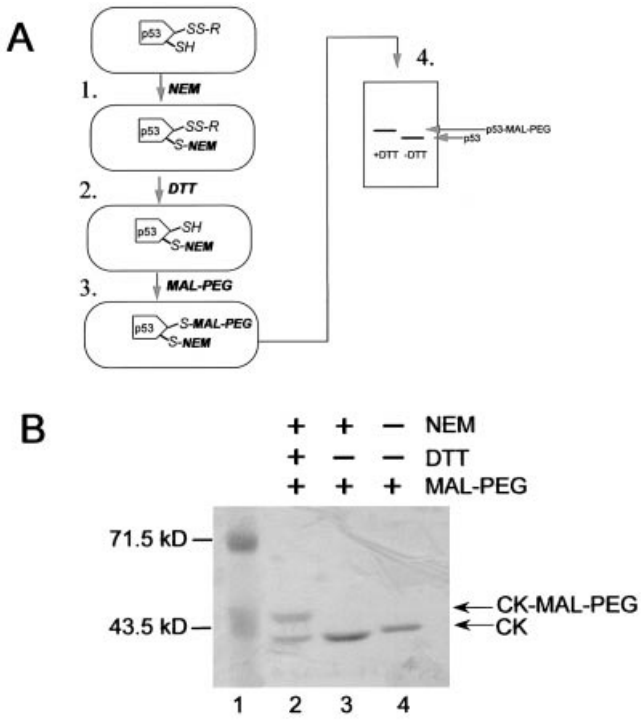

Figure 1 Tagging oxidized protein cysteine thiol groups with MAL-PEG

(**A**) Schematic diagram of the procedure for determining p53 cysteine oxidation status in cells (see the text for details). (**B**) Efficiency of MAL-PEG tagging procedure. Oxidized CK was treated with NEM, incubated with DTT and subsequently treated with MAL-PEG. Tagged CK was separated from untagged CK by SDS/PAGE and stained with Coomassie Blue. Lane 1, protein molecular-mass standards; lane 2, oxidized CK treated with NEM, DTT and MAL-PEG; lane 3, oxidized CK treated with NEM and MAL-PEG (no DTT reduction step); lane 4, oxidized CK treated with MAL-PEG (no NEM and no DTT). kD, kDa.

unreacted NEM. DTT was subsequently added to the lysate to reduce the oxidized thiol groups that were resistant to NEM derivatization. (Step 2). After DTT removal the soluble lysate was treated with MAL-PEG to form covalent adducts with free thiol groups, which were available as a result of DTT-mediated reduction (Step 3). MAL-PEG-treated soluble lysate was fractionated by SDS/PAGE (Step 4). If protein oxidation occurred in the cell then the electrophoretic mobility of the protein would be retarded due to the fact that it was covalently linked to MAL-PEG. An important control in this experiment was to process one sample without the addition of DTT (Step 2). In the absence of DTT-treatment oxidized protein thiol groups would be resistant to derivatization by MAL-PEG and the protein should exhibit no mobility-shift.

To assess the efficiency of the tagging procedure, a protein with a single oxidized cysteine residue was tested. CK only has one reactive cysteine residue on the surface of the protein [26]. Purified CK was oxidized (S-thiolated) with hydroxyethyl disulphide to form a mixed disulphide adduct on Cys^{283} [24]. Isoelectric focusing gel analysis [27] showed that greater than 90% of CK was oxidized with a stoichiometry of one mixed disulphide per protein molecule (results not shown). After treatment of the S-thiolated CK with excess NEM, to block maleimide-reactive sites, the protein was treated with DTT and tagged with MAL-PEG. Tagged CK was separated from untagged CK by SDS/PAGE and the gel was stained with Coomassie Blue. The MAL-PEG-treated CK contained a single retarded band (CK with attached MAL-PEG) that constituted approximately 54% of the total protein (Figure 1B). It was not possible to increase the amount of MAL-PEG-modified protein by simple changes in reaction conditions (i.e. pH, reaction time or temperature). The present data suggest that the MAL-PEG reagent was only capable of reacting with half of the available reactive thiol groups. Since it is known that CK is a dimer containing one reactive cysteine residue on each subunit, the rather large molecule added to the protein (MAL-PEG) may have prevented simultaneous modification of both reactive cysteines in the dimeric protein. Importantly Figure 1(B) shows that in the absence of the DTT step, oxidized CK failed to react with MAL-PEG. Thus in comparison with the isoelectric focusing method, the MAL-PEG tagging method is approximately 60% as efficient.

It is known that cells contain different forms of oxidized cysteine that are sensitive to reduction by DTT. These include protein disulphides, S-glutathiolated and S-cysteylated proteins, as well as S-nitrosylated proteins. Protein tagging with MAL-PEG did not differentiate between these various molecular modifications since the reduction step (addition of DTT) would lead to a reactive thiol that could be modified by MAL-PEG in each case.

PDTC treatment oxidizes a significant proportion of p53 in MCF7 cells

Previously, we showed that PDTC treatment oxidizes a temperature-sensitive p53 mutant in cultured rat cells [17]. Mutant p53 proteins often display altered conformations [28]. Thus a major question raised from our previous work is whether endogenous, wild-type p53 in cultured cells is susceptible to oxidation and, if so, what is the stoichiometry of oxidation? Due to the low abundance of wild-type p53 these questions could not be directly addressed with the oxidation detection method we previously employed (results not shown). The MAL-PEG tagging method was developed to determine if wild-type p53 was susceptible to PDTC-mediated oxidation. MCF7 breast cancer cells were incubated with either 60 μM or 120 μM PDTC for 5 h. After tagging oxidized cysteine residues on soluble cellular proteins with MAL-PEG, proteins were separated by SDS/PAGE and electroblotted. The p53 protein was detected by Western-blot hybridization. Figure 2(A) shows that a significant proportion of p53 (approximately 25%) was tagged after treatment with 60 μM PDTC. Prior to PDTC treatment approximately 3–12% of the p53 was oxidized. When the concentration of PDTC was increased to 120 μM, the percentage of tagged p53 was maintained at 25%. To investigate the kinetics of p53 oxidation, cells were treated with 60 μM PDTC for up to 6 h. Figure 2(B) shows that the percentage of oxidized p53 increased dramatically between 1 and 2 h of continuous PDTC treatment. There was no further increase in the percentage of total p53 that was oxidized between 2 and 6 h of treatment. Since the data with CK showed that MAL-PEG may not react with all free thiol groups in a protein, the data presented here suggest that 25% or more of the p53 was oxidized to a DTT-sensitive form by PDTC treatment.

The data in Figure 2(A) show that PDTC also led to a 3-fold increase in the overall level of p53. Many stresses lead to p53 protein induction, including oxidizing reagents, such as hydrogen peroxide and hypochlorous acid [29,30]. Recent reports show that the p53-specific antibody used in our Western-blot analysis (DO-1) binds to a region of p53 that is sensitive to alterations in phosphorylation at Ser^{20} [31–33]. These reports showed that the DO-1 antibody fails to bind when p53 is phosphorylated at Ser^{20}

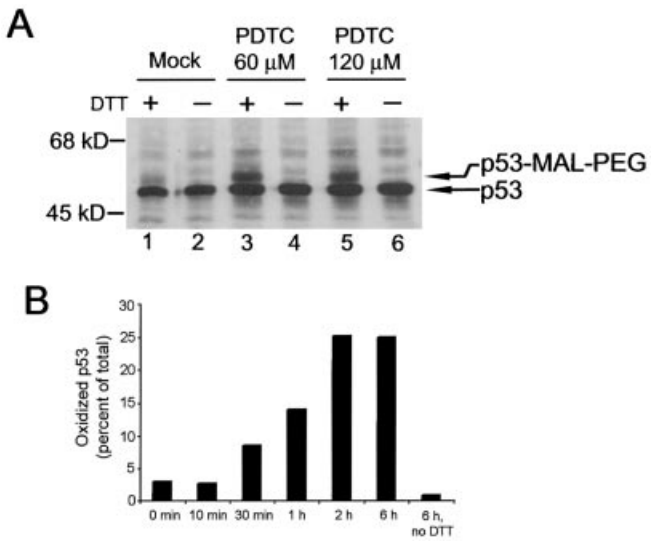

Figure 2 PDTC treatment of MCF7 cells led to increased levels of p53 oxidation

(**A**) Cells were treated with 60 μM or 120 μM PDTC for 5 h or mock-treated with vehicle control. Cell lysates were processed for p53 oxidation detection according to the protocol in Figure 1. Following SDS/PAGE cellular proteins were electroblotted on to PVDF paper. The p53 protein was detected with the DO-1 antibody followed by peroxidase-conjugated goat anti-mouse IgG. The amount of lysate protein loaded in each lane was 25 μg. Lanes 1, 3 and 5 included the DTT treatment step. Lanes 2, 4 and 6 excluded the DTT treatment step. MAL-PEG-conjugated p53 and unreacted p53 are shown. (**B**) Cells were treated with 60 μM PDTC and processed for detection of oxidized p53 at the indicated times. Densitometry was used to determine the percentage of total p53 that was oxidized p53. The maximum amount of oxidized p53 (at 2 h) was 25% and the minimum amount of oxidized p53 (a blank lane) was set to 0%.

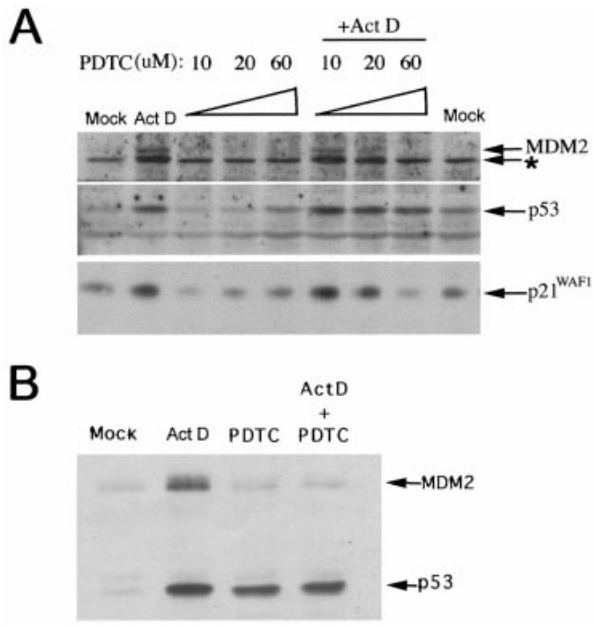

Figure 3 Treatment of MCF7 cells with PDTC inhibits actinomycin D-mediated up-regulation of MDM2 and p21$^{WAF1/CIP1}$

(**A**) Cells were treated with either 16 nM actinomycin D (Act D) in the presence of the indicated PDTC concentrations or with PDTC alone. Soluble whole-cell lysates (25 μg of protein) from harvested cells were applied to each lane. The p53 protein, MDM2 and p21$^{WAF1/CIP1}$ protein were detected by Western blotting using appropriate antibodies (p53, DO-1; MDM2, 2A10 plus SMP14; p21, Ab-1). The asterisk denotes 2A10 cross-reactive protein. (**B**) Cells were treated with 16 nM Act D, 60 μM PDTC or both for 5 h prior to harvesting. Western blot analysis was carried out as in (**A**) except that the only antibody used to detect MDM2 was SMP14. No analysis of p21$^{WAF1/CIP1}$ was carried out.

and that a change in phosphorylation occurs at this site when cells are treated with certain stresses. Thus it is possible that the increase in DO-1 reactivity we observed after PDTC treatment represented net dephosphorylation of Ser^{20} within p53. It is also possible that p53 in these cells may be oxidized in response to stresses not involved in oxidation. To test this, we investigated whether p53 was oxidized after treatment of cells with actinomycin D, a DNA-damaging agent that acts through topoisomerases to cleave double-stranded DNA. We found no evidence for p53 oxidation after treatment with 16 nM actinomycin D although p53 levels increased more than 3-fold (results not shown). On the other hand, after normalizing for protein level induction, treatment with PDTC led to a 2–3-fold increase in p53 oxidation relative to the vehicle-treated cells.

Previous studies have shown that, *in vitro*, p53 can form high-molecular-mass oligomers, which can be dissociated into monomers by DTT [14]. This opens up the possibility that oxidized p53 may form intersubunit disulphide bonds. To test this possibility, lysate from PDTC-treated MCF7 cells was separated on a denaturing non-reducing gel. The proteins were electroblotted and p53 protein was analysed by Western blot analysis. No higher molecular-mass forms of p53 were detected by this procedure (results not shown) suggesting that p53 does not form intersubunit disulphide linkages.

PDTC treatment prevents up-regulation of p53 responsive downstream effector genes

Some human tumours sustain mutations at cysteine residues that reside in the DNA binding domain of p53 [34]. This suggests that these cysteine residues are critical for proper tumour suppressor activity and that p53 oxidation might lead to loss of p53 function. It was previously shown that treatment of cells with PDTC inhibits p53 (derived from nuclear extracts) from binding to a p53-responsive DNA element *in vitro* [19]. If PDTC treatment oxidizes p53 it should also prevent p53 downstream effector gene activation. To test this, we asked whether PDTC prevented *MDM2* and *p21*$^{WAF1/CIP1}$ up-regulation in response to the DNA damaging agent, actinomycin D. Actinomycin D is a strong activator of p53 signal-transduction pathways [35,36]. Figure 3(A) shows that PDTC treatment inhibited actinomycin D-induced up-regulation of p21$^{WAF1/CIP1}$ protein and MDM2 in MCF7 cells in a dose-responsive manner. Because one of the MDM2 antibodies (2A10) appeared to react with a second protein of lower molecular-mass (denoted with an asterisk in Figure 3A) we re-assessed the ability of PDTC to prevent MDM2 up-regulation in response to actinomycin D using the highly specific MDM2 antibody, SMP14. Figure 3(B) shows that PDTC prevented MDM2 up-regulation in response to actinomycin D treatment. Interestingly, PDTC did not prevent the induction of p53 in response to actinomycin D. Furthermore, PDTC itself led to a slight induction of p21$^{WAF1/CIP1}$ protein. This may be due to the fact that PDTC treatment increases p21$^{WAF1/CIP1}$ protein levels via a p53-independent mechanism. Alternatively, p53 may not be completely oxidized and, due to the fact that its level is increased after PDTC treatment, it may be capable of weakly activating the *WAF1/CIP* promoter. Overall, the data

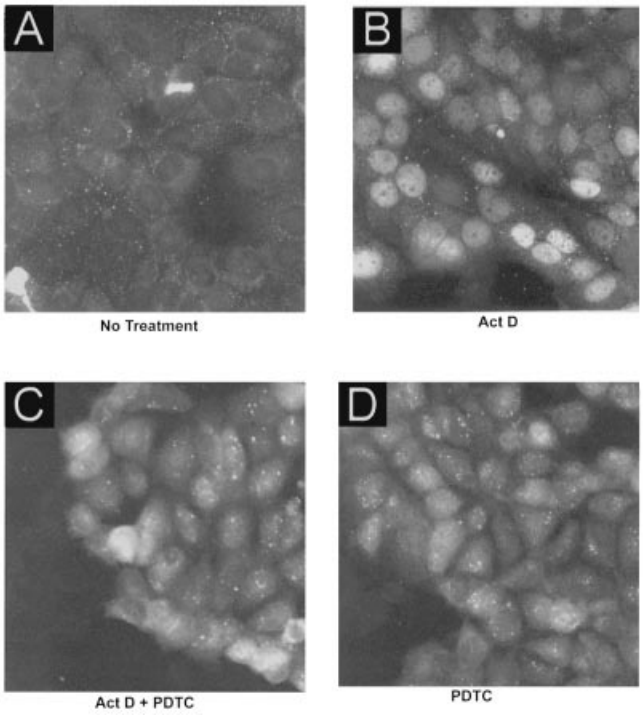

Figure 4 PDTC alters p53 nuclear accumulation properties

(**A**) MCF7 cells were vehicle-treated for 5 h. (**B**) Cells were treated with 16 nM actinomycin D (Act D) for 5 h. (**C**) Cells were treated with 16 nM Act D plus 60 μM PDTC for 5 h. (**D**) Cells were treated with 60 μM PDTC for 5 h. Fixed cells were treated with anti-p53 monoclonal antibody (1801) followed by FITC-conjugated goat anti-mouse antibody.

are consistent with the idea that PDTC oxidizes p53 at cysteine residues that affect the DNA binding domain of p53 and the idea that oxidation prevents p53 from efficiently transactivating certain p53 effector genes.

PDTC alters p53 nuclear accumulation properties

Previously, we showed that PDTC alters p53 nuclear accumulation properties in cells that express a temperature-sensitive p53 mutant [17]. Therefore we determined whether wild-type p53 nuclear accumulation properties in MCF7 cells were altered by PDTC. Figure 4 shows that the majority of p53 resided in the cytoplasm of proliferating MCF7 cells. After incubation with actinomycin D for 5 h, p53 accumulated in the nucleus. When MCF7 cells were treated with PDTC, p53 appeared to accumulate in both the nucleus and the cytoplasm. The two treatments led to different p53 subcellular accumulation properties. When PDTC and actinomycin D were added to the cells simultaneously, the p53 accumulation pattern appeared to be identical to the pattern observed with PDTC alone. The result suggests that PDTC treatment alters p53 subcellular localization properties normally associated with actinomycin D treatment. The fact that p53 did not efficiently accumulate in the nucleus after PDTC treatment may contribute to the fact that p53 fails to efficiently up-regulate MDM2 and $p21^{WAF1/CIP1}$.

DISCUSSION

A hypothesis has been put forward suggesting that p53 may be redox regulated [13]. Recently it was shown that the metal-chelating agent PDTC oxidizes p53 in cultured cells that overexpress a mutant form of the p53 protein [17]. This result suggests that PDTC may lead to oxidation of a mutant form of p53 that may have an altered conformation. It also leaves open the possibility that the oxidation is substoichiometric. In the present study we show that a significant proportion of endogenous wild-type p53 is oxidized in MCF7 cells after treatment with PDTC. A novel protein thiol-tagging agent was employed to demonstrate that at least 25% of p53 is oxidized after treatment of MCF7 cells with PDTC.

The method described in the present study may prove to be useful for detecting reversible oxidative modification of many different proteins. It may be especially useful to demonstrate oxidation of low-abundance proteins, such as transcription factors and signal-transduction components. Since the method is based on Western blots of SDS/PAGE gels, many different proteins can be studied by antibody detection techniques already in common use. In the case of p53, it was possible to study oxidation of the protein in cultured cells, without the need for overexpression of the protein of interest. However, because the method does not discriminate between the various potential forms of thiol oxidation (S-glutathiolation, S-cysteylation, protein disulphides and S-nitrosylation), it will be necessary to use other more laborious methods to identify the adduct on the susceptible cysteine thiol groups of p53.

Although the MCF7 cell line is derived from an adenocarcinoma of the breast it encodes a wild-type p53 gene. This was confirmed for the MCF7 cells used in the present study by sequencing exons 2–11 of the p53 gene (results not shown). Studies have shown that some downstream functions of the p53 signal-transduction pathway appear to be intact in MCF7 cells [37,38]. In the present study, PDTC treatment prevents actinomycin D-mediated up-regulation of the p53 downstream effector gene products, $p21^{WAF1/CIP1}$ and MDM2, at a concentration known to oxidize p53 inside the cell. Our results suggest that p53 is likely to be predominantly oxidized at one cysteine thiol group by dithiocarbamates.

Dithiocarbamates of biological significance include methyl dithiocarbamate (a herbicide) and ethylene bis(dithiocarbmate) (a fungicide also known as Nabam and Pestinal). The exact mechanism by which these dithiocarbamates act as selective toxins is unclear but, in the case of ethylene bis(dithiocarbamate), it is believed that this molecule can penetrate the plasma-membrane of fungi and kill these organisms through metal poisoning. Rats fed diets containing ethylene bis(dithiocarbamate) exhibit increased levels of copper in the central nervous system [39].

In line with the metal-loading properties of other dithiocarbamates, PDTC treatment of cultured cells leads to copper accumulation inside the cells [18–20]. *In vitro* experiments suggest that dithiocarbamates may be oxidized by Cu^{2+} to form thiuram disulphides [40]. This presents the possibility that thiuram disulphides may directly form a disulphide linkage with a susceptible p53 cysteine thiol group. It is also possible that the thiuram disulphides oxidize the intracellular antioxidant, glutathione. Glutathione may form a mixed disulphide with PDTC or form glutathione disulphide. The oxidized glutathione may then go on to perform a disulphide exchange reaction with a cysteine thiol group present on p53. Indeed, PDTC treatment of cultured cells leads to a dramatic increase in the glutathione disulphide/glutathione ratio in the cell [18,20]. Since there was no evidence

for intersubunit disulphides of p53 in our experiments, either an internal disulphide or an S-glutathiolated form of p53 may be formed during PDTC treatment.

Our results indicate that PDTC-treatment oxidized at least 25% of the p53 protein in the cell. We used MAL-PEG as the oxidation detection agent to make this estimate. It is possible that our p53 tagging procedure is not 100% efficient. Indeed, when our tagging agent was used to detect a mixed disulphide formed between 2-mercaptoethanol and CK it proved to be only 54% efficient. CK forms a homodimer [41]. It is possible that one MAL-PEG adduct reacts with a single subunit of the CK dimer. One MAL-PEG adduct may prevent the free thiol group of the second CK subunit from reacting with another MAL-PEG molecule either by steric hindrance or by altering the conformation of the CK dimer. The p53 protein forms a homotetramer in solution, but it is not known how the cysteine-containing domains are orientated with respect to one another. The efficiency of our tagging procedure, which may be different for p53 than for CK, opens the possibility that more than 25% of the p53 in PDTC-treated cells was oxidized.

Recent reports suggest that the p53 redox state is tightly controlled. Three proteins known to reduce protein disulphide bonds also appear to modulate p53 activity. Redox factor-1 (Ref-1) has been shown to increase p53 binding to DNA *in vitro* and increase p53-mediated transactivation of some of its promoters including the $p21^{WAF1/CIP1}$ promoter [42,43]. Thioredoxin reductase 1 ('TRR1') is required for human p53 transactivation activity in two yeast species [44,45]. Thioredoxin, a small protein substrate for thioredoxin reductase, which can reduce protein disulphide linkages, has also been shown to increase p53-mediated transactivation [46]. Thioredoxin appears to act in conjunction with Ref-1 to up-regulate p53 DNA binding activity. The fact that these potentially redox-modulating proteins up-regulate p53 activity suggests that p53 redox status requires tight regulation. In the future, it will be necessary to determine the site of p53 oxidation and the mechanism by which cysteine residues become oxidized. Finally, it will be important to assess the contribution of p53 oxidation to pathological conditions in humans.

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