

# Induction of Gene Amplification as a Gain-of-Function Phenotype of Mutant p53 Proteins<sup>1</sup>

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## ABSTRACT

Gene amplification accompanies tumor progression and is involved in the development of drug resistance. Previously, we reported (A. Albor *et al.*, *Cancer Res.* 58: 2091–2094, 1998) that mutant p53 proteins conserve the capacity to interact with and activate topoisomerase I, and that this could be a mechanism for induction of genomic instability by mutant p53 proteins. To test this hypothesis, the effect of exogenous mutant p53 protein expression on genomic instability in human p53<sup>-/-</sup> Saos-2 cells was measured by the frequency of formation of *N*-(phosphoacetyl)-L-aspartate (PALA)-resistant (PALA<sup>R</sup>) colonies, mediated by gene amplification. Interaction of exogenous mutant p53 and topoisomerase I was confirmed by immunoprecipitation. Growth under continuous expression of mutant p53 proteins for 16–17 population doublings increased the frequency of appearance of PALA<sup>R</sup> colonies after subsequent exposure to PALA. Subtoxic concentrations of camptothecin (which stabilizes topoisomerase I cleavage complexes, mediating nonhomologous recombination) produced a dose-dependent increase in PALA<sup>R</sup> colonies, and combining expression of mutant p53 with exposure to camptothecin produced a greater than additive increase in PALA<sup>R</sup> colony formation. These results indicate that mutant p53 proteins promote gene amplification independently of their capacity to inactivate the wild-type p53 protein, and suggest that this effect is dependent on interaction of mutant p53 with topoisomerase I. Additional studies are needed to assess the potential of targeting mutant p53 interaction with topoisomerase I for the reduction of drug resistance development during chemotherapy.

## INTRODUCTION

Genomic stability requires cells to correctly replicate and segregate mitotic chromosomes, preventing sequence alterations and chromosomal aberrations (1). Cancer cells commonly have an increased mutation rate, both in DNA sequence and in chromosomal segregation (microsatellite and chromosomal instability, respectively; Ref. 2).

The p53 gene codes for a transcription factor that is posttranslationally induced after genotoxic damage, controlling the expression of genes involved in cell cycle arrest and apoptosis (3, 4). About 50% of human tumors contain a missense point mutation in one of the p53 alleles, often accompanied by inactivation of the wild-type allele (5, 6). Missense p53 mutant proteins are typically impaired in DNA binding and transactivation, and have increased protein stability, constitutively accumulating to high levels (7–11). Mutant p53 proteins can compromise the function of the wild-type allele by heterotetramerization, for instance, during oncogene-mediated transformation of p53<sup>+/+</sup> fibroblasts (12), but they also have an intrinsic oncogenic activity independent of wild-type protein inactivation, which explains the retention of mutant p53 expression when the wild-type allele has been inactivated by deletion or rearrangement (13). This gain-of-function activity is evident when mutant p53 is expressed in a

p53<sup>-/-</sup> background. Several p53 missense mutants induced proliferation, colony formation, and metastasis when introduced into p53<sup>-/-</sup> leukemia cells (14–16), tumorigenicity and soft agar growth in the p53<sup>-/-</sup> cells 10(1) and Saos-2 (17), and oncogenic activity *in vivo* in the mouse skin chemical carcinogenesis model (18–20).

Potential mechanisms of mutant p53 gain-of-function activity include aberrant transcriptional activities and increased genomic instability (13). Mutant p53 has been found to foster genomic instability in the absence of wild-type p53 (19, 21), which may be caused by interaction with other cellular proteins involved in the maintenance of chromosomal integrity. Topoisomerase I and p53 are interacting proteins (22), and wild-type p53 is required for recruitment of topoisomerase I into cleavable complexes after UV irradiation (23). We reported previously that mutant p53 proteins retain their capacity to interact with and activate topoisomerase I (24). Furthermore, in cells expressing a functional wild-type p53 protein, the interaction of the two proteins is tightly regulated after DNA damage, whereas in cells expressing p53 Arg<sup>273</sup> to His<sup>273</sup> mutant protein (H273), the interaction is constitutive (25). Topoisomerase I is a ubiquitously expressed protein that relaxes DNA supercoils generated during transcription and replication, by sequentially cutting and religating one DNA strand (26). If this process is interrupted (by the nearby presence of DNA damage or by incubation with specific topoisomerase I poisons such as camptothecin), free cleavage complexes are generated, consisting of double-strand breaks covalently linked to topoisomerase I. These complexes efficiently catalyze nonhomologous recombination in cell-free systems *in vitro* and *in vivo* (27–35). Because mutant p53 proteins are overexpressed in cancer cells, retention of topoisomerase I interaction with mutant p53 proteins could have a major impact on genomic instability. We examined the role of the mutant p53-topoisomerase I interaction in the promotion of genomic instability, as measured by gene amplification of *CAD*,<sup>3</sup> which mediates resistance to the metabolic inhibitor PALA (36). We have created derivatives of Saos-2 cells, a human p53<sup>-/-</sup> osteosarcoma cell line, with inducible expression of stably transfected mutant p53 genes. Under conditions in which mutant p53 and topoisomerase I interact in the cells, expression of mutant p53 proteins during multiple population doublings increased the frequency of genomic amplification on subsequent exposure of cells to PALA. Subtoxic concentrations of camptothecin produced a dose-dependent increase in the frequency of gene amplification, and concurrent expression of mutant p53 and incubation with camptothecin had a greater-than-additive effect in the frequency of gene amplification. These results implicate topoisomerase I in the mechanisms of gene amplification and indicate that the effects of mutant p53 and camptothecin on gene amplification are through a common mechanism. Our results imply that the interaction of mutant p53 with topoisomerase I may be responsible for the dominant gain-of-function oncogenic effects of mutant p53 proteins.

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<sup>3</sup> The abbreviations used are: *CAD*, carbamoyl-P-synthetase, aspartate transcarbamoylase, dihydroorotase tri-functional enzyme; Dox, doxycycline; GST, glutathione-S-transferase; PALA, *N*-(phosphoacetyl)-L-aspartate; PALA<sup>R</sup>, PALA-resistant.

## MATERIALS AND METHODS

**Reagents.** PALA was generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH (Bethesda, MD). Camptothecin and Dox were purchased from Sigma-Aldrich Co. (St. Louis, MO).

**Antibodies.** Scl70 serum (Immunovision, Inc., Springdale, AR) recognizes an epitope in the COOH-terminal half of the topoisomerase I protein (37).  $\alpha$ -1-140 is a polyclonal rabbit antiserum that we developed by immunization with the first 140 amino acid residues of human topoisomerase I. Antibodies DO-1 (p53), SWT3D1 (topoisomerase II), 1B5 (Hsp70), and C33 (c-myc) are mouse monoclonal antibodies, obtained from Oncogene Science, Cambridge, MA (DO-1, SWT3D1); Stressgen, Victoria, British Columbia, Canada (1B5); and Santa Cruz Biotechnologies, Inc., Santa Cruz, CA (C33).

**Generation of Saos-2 Cell Lines with Inducible Expression of Mutant p53 Proteins.** The cDNAs for S245- and H273-mutant p53 proteins were produced by site-directed mutagenesis as described previously (9). They were inserted into vector pTRE and stably transfected into tTA factor (38) expressing Saos-2 cells, to allow Dox-controlled expression (Clontech, Palo Alto, CA). Transfectants, selected with a blasticidin-resistance gene (Invitrogen, Carlsbad, CA), were expanded and tested for mutant p53 induction.

**Coimmunoprecipitation.** Cells were lysed in 50 mM HEPES (pH 7.8), 200 mM NaCl, 0.1% Triton X-100, and 20% glycerol. Crude protein extract (750–800  $\mu$ g) was incubated with either 10  $\mu$ l of a topoisomerase I-specific antiserum (Scl70 or rabbit  $\alpha$ -1-140) or 1  $\mu$ g of a human p53-specific antibody (DO-1). As a control, lysates were also incubated with rabbit preimmune serum or with IgG1. Protein-antibody complexes were collected with Sepharose-protein A beads, eluted with protein electrophoresis sample buffer [120 mM Tris (pH 6.8), 200 mM DTT, 20% glycerol, 4% SDS, 0.002% BPB], and separated by SDS-PAGE. Mutant p53, Hsp70, and topoisomerases I and II were detected by immunoblotting.

**In Vitro Protein-binding Assays.** Deletion mutants of the human topoisomerase I were produced by restriction digest of the full-length topoisomerase I cDNA (39), provided by Dr. J. C. Wang (Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA), and expressed in insect cells as histidine-tagged proteins (24). These cells were lysed in 50 mM Tris (pH 8.0), 1% NP40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50  $\mu$ M leupeptin, and 10  $\mu$ g/ml pepstatin A. Lysates were mixed with glutathione-Sepharose beads containing 500 ng/ $\mu$ l purified GST/p53 or GST/1-97 (24), and incubated for 30 min at 4°C. Beads were washed with 10 mM Tris (pH 7.9), 250 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 10% glycerol; were eluted with protein sample buffer; and were immunoblotted with an anti-histidine-tag antibody (Qiagen, Valencia, CA).

**PALAR Colony-formation Assays.** Individual groups of 200 cells/well were plated in 24-well plates in the absence or presence of 10 ng/ml Dox in the medium but in the absence of PALA. These groups were expanded to about 17 population doublings ( $\sim 2.5 \times 10^7$  cells), maintaining the same growth conditions. Cells were then plated at a cell density of  $2.5 \times 10^5$  cells per 150-mm tissue-culture dish, and allowed to attach for 48 h before the addition of PALA at nine times the ID<sub>50</sub> concentration ( $9 \times \text{ID}_{50}$ ). Whenever PALA was used, the medium contained dialyzed FCS to remove metabolic precursors from the medium that could interfere with the toxicity of PALA. Cells were kept in the presence of PALA until resistant colonies were visible. Colonies were fixed with methanol, stained with Giemsa, and counted. The ID<sub>50</sub> for each clone with or without mutant p53 induction was determined by standard growth curves. Cells ( $8 \times 10^4$ ) were seeded on 60-mm tissue-culture dishes in triplicate; and 48 h later, different concentrations of PALA were added. After 7 days, cells were washed with PBS and lysed [50 mM HEPES (pH 7.8), 500 mM NaCl, 0.1% Triton X-100, and 20% glycerol], and protein concentration was determined by the Bradford assay. Each concentration point was determined by experimental triplicates. The sensitivity to camptothecin was determined using the same procedure.

**Analysis of Gene Amplification.** Cells from PALAR colonies (expanded in medium with  $9 \times \text{ID}_{50}$  PALA used for selection) and the original PALA-sensitive clones were lysed in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 0.5% SDS, and were repeatedly extracted with phenol:chloroform:isoamyl alcohol. High molecular weight genomic DNA was then precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Purified DNA (10  $\mu$ g) was digested with *Hind*III and blotted with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled human *CAD* gene probes (from plasmids 1875033 and 1409043; American Type Culture Collection) or a human *hmRNP A1* gene probe as a control (provided by Dr. G. Dreyfuss, Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA). Radioactive signals were visualized by phosphorimaging.

## RESULTS

**Mutant p53 and Topoisomerase I Interact in Cells, Requiring the Central Domain of Topoisomerase I.** The p53<sup>-/-</sup> Saos-2 cells were stably transfected with plasmids for inducible expression of Gly<sup>245</sup> to Ser<sup>245</sup> (S245) or Arg<sup>273</sup> to His<sup>273</sup> (H273) human p53 mutants. Transfected clones were selected and tested for inducible and tightly regulated expression of mutant p53 proteins (Fig. 1A). A clone transfected with an empty expression vector (TRE.3) was also selected to be used as a control. Clones S245.3, S243.13, and H273.14 had very little expression of mutant p53 in the presence of Dox, but readily accumulated the p53 protein after Dox removal. These clones were selected and used in the experiments presented in this study.

The interaction of S245 and H273 with topoisomerase I in these cells was tested by coimmunoprecipitation. Topoisomerase I was immunoprecipitated with two independent antisera for human topoisomerase I, Scl70 and rabbit  $\alpha$ -1-140, specific for epitopes in the COOH- and NH<sub>2</sub>-terminal ends of topoisomerase I, respectively. Lysates were prepared with or without the induction of mutant p53. The S245 and H273 proteins were readily detected in Scl70 and  $\alpha$ -1-140 immunoprecipitates from induced cells (Fig. 1B–D). No p53 protein was immunoprecipitated with IgG1 or control preimmune serum. As a comparison, we tested the coimmunoprecipitation of mutant p53 with topoisomerase II and Hsp70 (Fig. 2). Both wild-type and mutant p53 proteins have been reported to interact with topoisomerase II  $\alpha$  and  $\beta$  through the COOH terminus of p53 (40, 41), whereas Hsp70 interacts only with mutant p53 proteins (42). Lysates from S245-expressing cells, with or without induction, were immunoprecipitated with human p53-specific monoclonal antibody DO1, and the presence of topoisomerase II and Hsp70 was analyzed by immunoblotting (Fig. 2). Surprisingly, topoisomerase II was readily detected only in the immunoprecipitates from cells grown in the presence of Dox, when only a small background of mutant p53 expression can be detected. In contrast, higher levels of the Hsp70 protein were immunoprecipitated from cells grown in the absence of Dox, when the levels of mutant p53 are higher. This result was consistent in three independent experiments and implies that any effects observed when mutant p53 protein is induced in Saos-2 cells are not attributable to interaction with topoisomerase II.

We have previously reported that the interaction between p53 and topoisomerase I requires the COOH-terminal domain of p53 (24). To determine the interacting domain within the topoisomerase I protein, deletion mutants of human topoisomerase I were also created and tested in binding assays (Fig. 3A). In Fig. 3B, the left panel (Lanes 1–4) shows the input topoisomerase proteins; the middle and right panels (Lanes 5–18) show the topoisomerase proteins retained by the GST fusion proteins. Only the full-length topoisomerase sequence (Fig. 3, *Topo I*) and the partial topoisomerase sequence “Top 1-484” form were trapped by GST/p53 (Fig. 3B, Lanes 7 and 9, respectively). GST alone or GST/1-97 [a GST fusion to the NH<sub>2</sub>-terminal domain of p53 (Fig. 3B, Lanes 14 and 17)] did not trap any of the topoisomerase I forms. These results indicate that interaction with p53 requires amino acid residues 140–484 of topoisomerase I.

**Continuous Expression of Mutant p53 Increases the Frequency of Gene Amplification.** We next examined whether mutant p53 proteins could increase gene amplification frequency. No significant

Fig. 1. Topoisomerase I and exogenous mutant p53 interact in stably transfected *p53*<sup>-/-</sup> Saos-2 cells. **A**, inducible expression of mutant p53 in stably transfected cell lines. Mutant p53 proteins were detected by immunoblotting with DO-1, 24 h after transfer to a medium without Dox (*Dox.*). *Arrowhead*, an endogenous *M<sub>r</sub>* 45,000 protein that cross-reacts with the antibody and is present in all conditions. **B**, coimmunoprecipitation of S245 with topoisomerase I (*Topo I*). S245.3 cells were lysed 24 h after induction of the mutant p53 protein, and topoisomerase I immunoprecipitated with Scl70. IgG1 was used as a control. Topoisomerase I and S245 were detected in 20  $\mu$ g of the crude protein extract (lysate) or the immunoprecipitates (*immunoprecip.*) by immunoblotting with Scl70 and DO-1. **C**, same as in **B**, but using rabbit  $\alpha$ -1-140 to detect immunoprecipitates topoisomerase I. Pre-immune rabbit serum is a negative control. **D**, coimmunoprecipitation of H273 with topoisomerase I. Lysates from H273.14 cells were treated as in **C**.

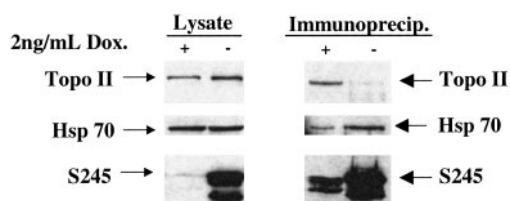
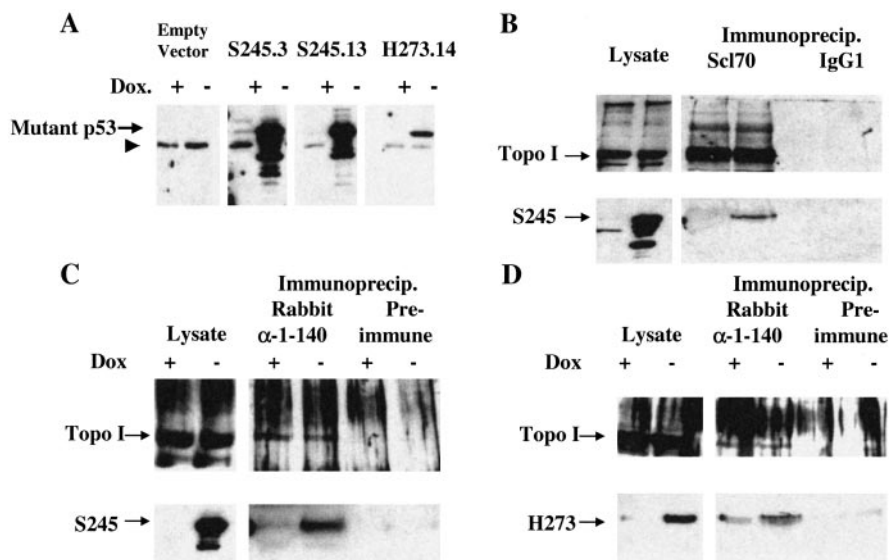


Fig. 2. Overexpressed mutant p53 does not interact with topoisomerase II (*Topo II*). Protein extracts were prepared from S245-expressing cells grown with or without mutant p53 induction (- or + *Dox.*, respectively). Crude lysates were immunoblotted using monoclonal antibodies SWT3D1 (*Topo II*), 1B5 (*Hsp70*), and DO-1 (*S245*). For immunoprecipitation, lysates were incubated with DO-1, and protein-antibody complexes were immunoblotted to SWT3D1, 1B5, and DO-1.

differences in PALA toxicity were found in the TRE.3, S245.3, S245.13, and H273.14 cell lines when incubated with or without Dox, with  $ID_{50} \sim 30 \mu M$  (Fig. 4, *inset*). Each cell line was then grown for 16 population doublings in the presence or absence of mutant p53 expression (Fig. 4). The frequency of PALA<sup>R</sup> colonies in the control cell line TRE was not affected by the presence or absence of Dox. However, expression of mutant p53 resulted in a 10- to 25-fold increase in the frequency of PALA<sup>R</sup> colonies. This effect was not caused by an increased plating efficiency in the presence of mutant p53 expression (Table 1).

The PALA<sup>R</sup> phenotype was attributable to amplification of the *CAD* gene, as shown by the increased *CAD* gene copy number in resistant relative-to-the-parental sensitive clone (Fig. 5). After quantification of the radioactive signals and normalization to hnRNPA1, 84% (21 of 25) of the clones had amplified *CAD* gene (Table 2), with an average increase in copy number of  $3.4 \pm 1.5$  fold. This value is consistent with the published values for single-step PALA<sup>R</sup> selection and is relevant for drug resistance (43).

We next determined whether the effect of mutant p53 on gene amplification frequency might be caused by the induction of c-myc, a transcription factor that can increase genomic instability and whose gene promoter has been reported to be induced by mutant p53 (13, 44-46). However, total c-myc protein steady-state levels did not change after the induction of S245 mutant p53 protein (Fig. 6), which indicated that this mechanism is unlikely.

**Subtoxic Concentrations of Camptothecin Increase Gene Amplification Frequency, and Concurrent Mutant p53 Expression Produces a Supra-additive Increase.** If topoisomerase I is able to mediate nonhomologous recombination events required for gene am-

plification, treatments that increase the stability of covalent topoisomerase I-DNA cleavage complexes, such as incubation with camptothecin, should promote gene amplification. To test this possibility, TRE.3 cells were expanded for 16-17 population doublings in the presence of 0-20 nM camptothecin, and the frequency of PALA<sup>R</sup> colonies was determined (Fig. 7A). The selected concentrations of camptothecin (5, 10, and 20 nM) produced no detectable reduction in cell proliferation (Fig. 7A, *inset*). Camptothecin produced a significant dose-dependent increase in the frequency of formation of PALA<sup>R</sup> colonies (from  $1.9 \pm 0.7$  colonies/ $10^5$  cells in the absence of camptothecin to  $13.8 \pm 5.5$  colonies/ $10^5$  cells in the presence of 20 nM camptothecin). These results imply a mechanism of gene amplification through topoisomerase I. We next determined the effect of

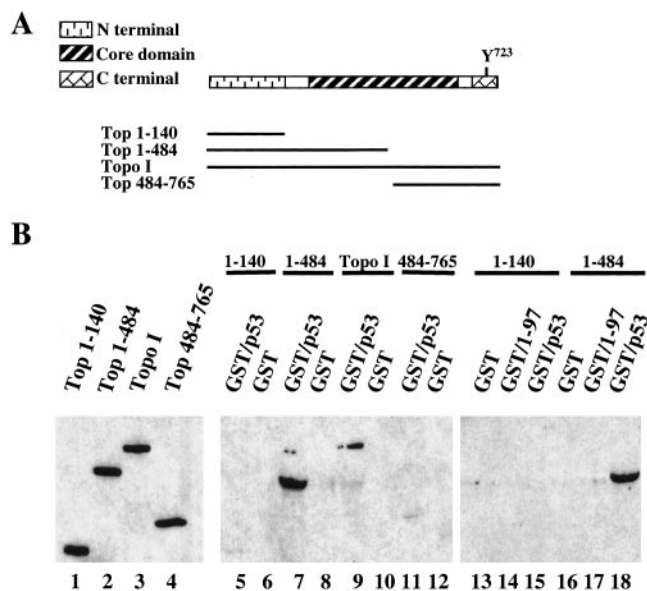


Fig. 3. Interaction with p53 requires the NH<sub>2</sub>-terminal half of the central core domain of topoisomerase I. **A**, schematic representation of the topoisomerase I deletion forms. Y<sup>723</sup>, the catalytic Tyr residue. *Topo I*, the full-length protein, residues 1-765. **B**, binding of topoisomerase I deletion forms to p53. Hexa-histidine-tagged topoisomerase I proteins were bound to purified GST or GST/p53 proteins, eluted with 2% SDS sample buffer, and detected by immunoblotting with an anti-hexa-histidine antibody (Lanes 5-18). Lanes 1-4, the initial input of topoisomerase I proteins. A GST fusion to the NH<sub>2</sub> terminus of p53 (*GST/1-97*, Lanes 14 and 17) was included in the reactions (Lanes 13-18) with topoisomerase I 1-140 (*Top 1-140*) and topoisomerase I 1-484 (*Top 1-484*).

Fig. 4. Expression of mutant p53 proteins S245 and H273 increases the frequency of gene amplification in Saos-2 cells. Cells from clones TRE.3, H273.14, S245.3, and S245.13 were expanded in the constant presence or absence of mutant p53 but in the absence of PALA. Cells were then plated and exposed to a  $9 \times ID_{50}$  concentration of PALA. After 5 weeks, resistant colonies were fixed, stained, and counted. Columns, the average of four replicates; error bars, mean SD; \*\* on top of the bars,  $P < 0.05$  statistical significant difference relative to the matched plus Dox (+dox) control (by Mann-Whitney-Wilcoxon test). Inset, growth curves of the four different cell lines in the presence of PALA. Cells were plated at the same density to be used in the PALA<sup>R</sup> colony formation assay, in the presence or absence of Dox and were exposed to PALA (0–800  $\mu$ M) for 1 week and lysed. Cell growth was estimated by measuring protein concentration in the lysates. Results are normalized relative to the control plates without PALA. Each point, the average of triplicate experiments plus the SD of the mean.

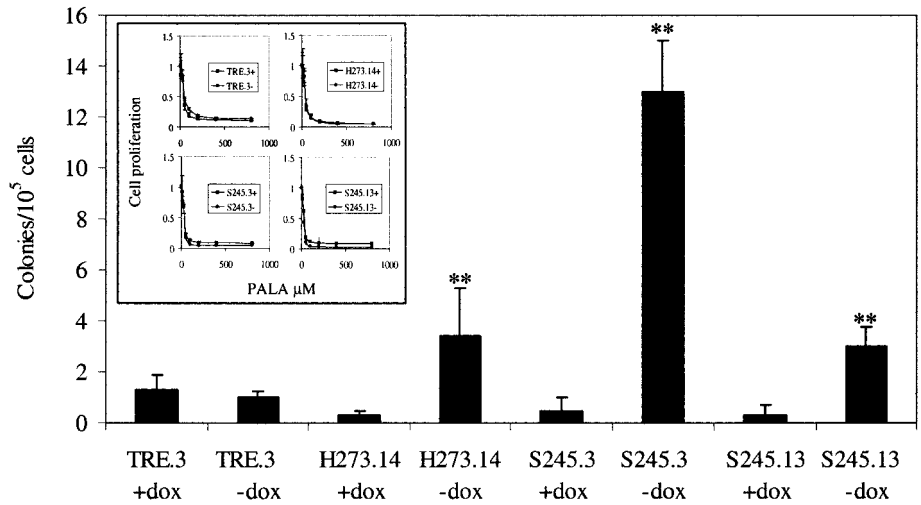


Table 1 Plating efficiency of Saos-2 cell lines expressing mutant p53 proteins

Cell line	Dox	Mutant p53 expression	% plating efficiency (n = 3) <sup>a</sup>
TRE	+	–	N.D. <sup>b</sup>
	–	–	N.D.
H273-14	+	–	17 ± 2.00
	–	+	20 ± 1.70 <sup>c</sup>
S245-3	+	–	33 ± 0.25
	–	+	28 ± 1.80 <sup>c</sup>
S245-13	+	–	20 ± 0.46
	–	+	22 ± 2.10 <sup>c</sup>

<sup>a</sup> Plating density was 3000 cells per 60-mm petri dish, in the presence or absence of Dox. After plating, cells were allowed to attach for 48 h; then PALA was added at  $9 \times ID_{50}$  concentration. After 4 weeks, colonies were fixed with methanol, stained with Giemsa, and counted. Results are expressed as mean ± SD.

<sup>b</sup> N.D., not done.

<sup>c</sup> Not statistically significant relative to the matched Dox+ condition.

combined camptothecin exposure and mutant p53 expression on gene amplification frequency (Fig. 7B). The presence of S245 mutant p53 expression did not produce any significant difference in the toxicity of camptothecin (Fig. 7B, inset). When S245.3 cells were expanded in the presence of 20 nM camptothecin but without mutant p53 expression, *CAD* gene amplification frequency increased from  $0.36 \pm 0.56$  to  $2.57 \pm 0.72$  colonies/10<sup>5</sup> cells. In the presence of mutant p53 expression, 20 nM camptothecin increased the frequency from  $4.48 \pm 0.98$  to  $11.33 \pm 1.27$  colonies/10<sup>5</sup> cells. This increase was more than double what would be expected if incubation with camptothecin had an additive effect. If mutant p53 and camptothecin

increased the frequency of gene amplification through independent mechanisms, their effects on gene amplification frequency would be additive. The supra-additive effect suggests that mutant p53 and camptothecin rely on a common mechanism through topoisomerase I. Taken together, these results suggest that enhancement of topoisomerase I activity at sites of DNA damage by mutant p53 proteins increases the likelihood of genetic recombination and the frequency of gene amplification events.

DISCUSSION

Wild-type p53 maintains chromosomal stability by controlling the growth arrest and apoptotic responses after a genotoxic stimulus. Although loss of wild-type p53 makes cells permissive for chromosomal instability (47–51), our results demonstrate that the expression of oncogenic mutant p53 proteins further increases the frequency of gene amplification by 10- to 25-fold in human cancer cells (Saos-2)

Table 2 Amplification of *CAD* in PALA<sup>R</sup> Saos-2 clones

Clone	Mutant p53 expression	Amplified <i>CAD</i> /total colonies analyzed <sup>a</sup>
TRE	–	2/2
H273-14	–	2/2
H273-14	+	5/5
S245-3	–	2/2
S245-3	+	4/5
S245-13	–	2/4
S245-13	+	4/5

<sup>a</sup> PALA<sup>R</sup> colonies were individually trypsinized using cloning rings and expanded in the presence of the selective PALA concentration. Genomic DNA was isolated, and amplification of the *CAD* gene was analyzed by Southern hybridization as explained in the “Materials and Methods” section.

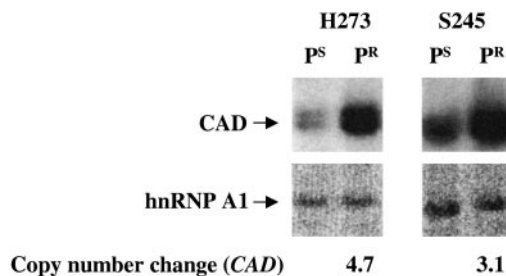


Fig. 5. Amplification of the *CAD* gene in PALA<sup>R</sup> colonies. Colonies were lifted and expanded in the presence of PALA ( $9 \times ID_{50}$ ). Genomic DNA was extracted from either PALA<sup>R</sup> colonies (P<sup>R</sup>) or the original cell line with inducible expression of the indicated human mutant p53 (P<sup>S</sup>). The *CAD* and *hnRNP A1* genes were detected by Southern blotting, using consecutive hybridization to cDNA probes for each gene. Radioactive signals were quantified using a Packard-Bell phosphorimager. The *hnRNP A1* signal was used to normalize the *CAD* gene signals. *CAD* gene copy number change in P<sup>R</sup> is expressed as *n*-fold, relative to P<sup>S</sup>. (See Table 2 for a summary of all of the analyzed colonies and the results.)

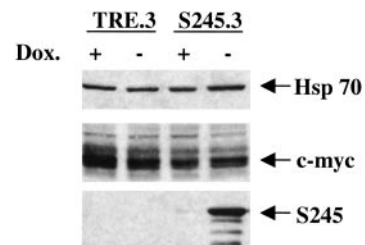


Fig. 6. S245 mutant p53 protein does not induce c-myc protein expression. Lysates were prepared from S245.3 cells and the control cells, TRE.3, and grown in the presence or absence of Dox. For each lysate, 20  $\mu$ g of total protein were loaded in a SDS-PAGE gel, and sequentially immunoblotted with antibodies specific for human p53 (DO1), c-myc (C33) and Hsc 70 (1B5).

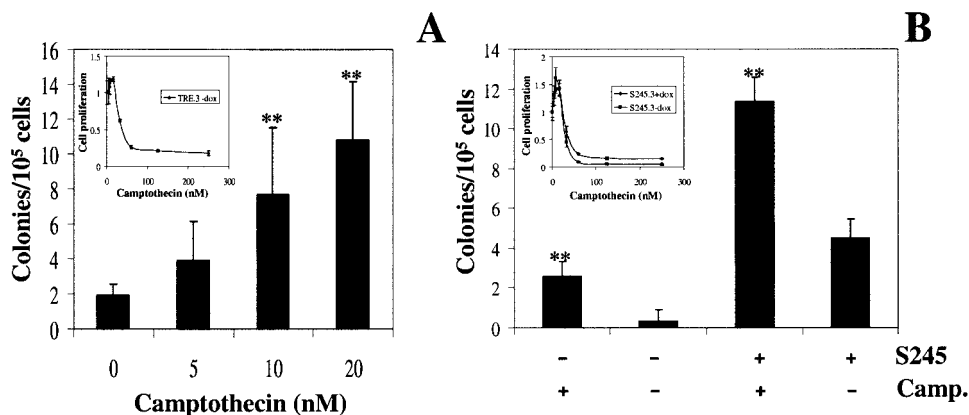


Fig. 7. Mutant p53 and camptothecin induce a greater-than-additive increase in the frequency of gene amplification. *A*, to measure the effect of camptothecin alone, Saos-2 cells were expanded under the constant presence of the indicated concentration of camptothecin, but in the absence of PALA. Then, resistant colonies were selected with  $9 \times ID_{50}$  PALA, Giemsa-stained, and counted. Each bar, the average of five replicates plus the SD of the mean. \*\*,  $P < 0.05$  relative to control (by Mann-Whitney-Wilcoxon test). The low concentrations of camptothecin used did not produce detectable inhibition of cell growth (*inset*), as determined by toxicity assays performed as in Fig. 4. *B*, to measure the effect of combined mutant p53 expression and camptothecin, S245.3 cells were expanded under the constant presence of 20 nM camptothecin (*Camp.*) and/or S245 mutant p53 expression, but in the absence of PALA. Then, PALA<sup>R</sup> colonies were selected and counted as in *A*. Each bar, the average of five replicates plus the SD of the mean. \*\*,  $P < 0.05$  relative to matched control without camptothecin (by Mann-Whitney-Wilcoxon test). Expression of mutant p53 did not produce a detectable effect on the  $ID_{50}$  to camptothecin (*inset*), as determined by toxicity assays performed as in Fig. 4.

that are already permissive because of their  $p53^{-/-}$  status. During tumorigenic progression, gene amplification is a mechanism of oncogene overexpression and drug resistance (52–55). Our results could explain the persistence of mutant p53 expression in human cancers even when the other allele has been lost by deletion or rearrangement (5, 6, 56, 57), because cells with mutant p53 expression would be more likely to develop drug resistance and to undergo additional genetic changes required for increased malignancy.

We observed differences in the extent of the effect among the three mutant p53 clones used (S245.3, S245.13, and H273.14). This could be caused by clonal variability by which other cellular factors may affect genomic instability. Comparing S245 and H273 mutants, we found that the levels of mutant S245 reached after Dox removal are higher than H273 mutant, but H273.14 reached levels of gene amplification similar to those of S245.13. Whether this is caused by higher activity of the H273 mutant or by clonal variability between S245.13 and H273.14 cannot be assessed based on our observations, and it is currently under investigation.

Greater-than-additive induction of gene amplification by combined mutant p53 expression and camptothecin exposure suggests the involvement of mutant p53 interaction with topoisomerase I. Two possibilities might explain how the interaction between these two proteins could increase gene amplification frequency. First, interaction with mutant p53 could favor the interstrand ligation activity of covalent DNA-topoisomerase I cleavage complexes at sites of DNA damage. This could be attributable either to a direct increase of topoisomerase I ligation activity (24) or to the ability of mutant p53 to facilitate the alignment of the DNA molecules before ligation, through the binding of either short stretches of single-stranded DNA or the protruding ends (even only two nucleotides) of double-stranded DNA (58, 59). This activity is mediated by the COOH-terminal domain of p53 even in isolation, and it is likely to be preserved in p53 proteins mutated in the central-sequence-specific DNA binding domain (such as the mutants found in human cancers and used in these experiments). Second, changes in mutant p53 transcriptional activity, thereby altering patterns of gene expression, could be brought about by interaction with topoisomerase I, because of the participation of this enzyme in the assembly of basal factors during transcriptional initiation (60, 61). Mutant p53 can increase transcription from a number of gene promoters (13), in particular *c-myc*, which fosters genomic instability (44, 46). However, there was no detectable induc-

tion of *c-myc* protein after mutant p53 induction in the Saos-2 cells. Additional studies are needed to determine the role of mutant p53 transcriptional activation on the induction of gene amplification.

Interaction of mutant p53 protein with topoisomerase II as a possible mechanism was eliminated after detecting an interaction with topoisomerase II only under noninduction conditions, when very small background levels of the mutant p53 protein were present. A possible explanation for this result is differences in posttranslational modifications of the mutant p53 protein under induction or noninduction conditions. Wild-type p53 protein is subject to Ser/Thr phosphorylation and other posttranslational modifications in its NH<sub>2</sub>- and COOH-terminal domains (3). The way in which mutant p53 proteins may be posttranslationally modified is unknown. It is possible that differences in posttranslational modifications between mutant S245 p53 protein at low, background expression and at high, overexpressed levels may alter the affinity of mutant p53 for topoisomerase II and account for this result.

Although our results demonstrate that mutant p53 induces gene amplification, they are not conclusive as to whether this effect is responsible for the gain-of-function phenotypes previously observed by others (*e.g.*, growth in soft agar *in vitro* and tumorigenicity *in vivo*). However, an increased frequency of gene amplification and, presumably, genomic rearrangements, would make a cancer cell more likely to develop resistance to chemotherapeutic drugs. Our results indicate that amino acids 141–484 of topoisomerase I are involved in the interaction, whereas p53 protein interacts through amino acids 299–390 (in mice; equivalent to 302–393 in humans; Ref. 24). Our ongoing work includes further delimiting the interacting domains on both proteins, and the cocrystallization of these domains. Future studies will determine the feasibility of the structure-based design of small diffusible molecules inhibitory of their interaction (62), as a strategy to reduce or prevent the development of drug resistance.

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## Induction of Gene Amplification as a Gain-of-Function Phenotype of Mutant p53 Proteins

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