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DNA damage induced p53 stabilization: no indication for an involvement of p53 phosphorylation

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Abundance and activity of p53 are predominantly regulated posttranslationally. Structural disturbance in transcribed genes induced by radiation, e.g. DNA damage, or by transcriptional inhibitors cause p53 protein stabilization by a yet unknown mechanism. Using stable and transient transfections for the analysis of p53 mutant proteins, we have ruled out a role in stabilization by UV, gamma irradiation or actinomycin C for the following putative phosphorylation sites in the p53 protein: serines 6, 9, 15, 33, 315 and 392, and threonine 18. By double mutation combinations of phosphorylations were also ruled out; 6,9; 15,18; 15,37. These mutations eliminate modifications by casein kinases I and II, DNA-PK, ATM, CDK and JNK. Also the 30 carboxyterminal amino acids are not required for induced p53 stabilization. Thus neither phosphorylations of individual amino acids nor interactions of the carboxyterminus of p53 with cellular macromolecules appear to play a role in the stabilization process. The only single prerequisite for induced stabilization of p53 is its prior destabilization by Mdm2. However, the level of active Mdm2 must be controlled carefully: overexpression of Mdm2 inhibits UV induced p53 stabilization.

Keywords: ultraviolet irradiation; gamma irradiation; actinomycin D; p53 stabilization

Introduction

The amount and the activity of the cell cycle surveillance protein p53 are regulated predominantly by posttranslational mechanisms (Maltzman and Czyzyk, 1984; Kastan *et al*., 1991; Fritsche *et al*., 1993; Lu and Lane, 1993; reviewed in Meek, 1994). The active protein serves as a transcription factor which targets for instance, cell cycle control genes, and may participate in DNA repair as it has been detected in association with the TFIIH complex (Wang *et al*., 1994, 1995; Xiao *et al*., 1994). The recognition of target promoter sequences involves the central domain of the p53 protein while the N- and C-terminal sequences appear to exert intra- and intermolecular regulatory functions (reviewed in Gottlieb and Oren, 1996; Ko and Prives, 1996). Activation and stabilization of p53 are triggered by agents that damage DNA, and it appears that p53 binds to damaged DNA via its C-terminus (Foord *et al*., 1991; Bayle *et al*., 1995; Lee *et al*., 1995; Jayaraman and Prives, 1995). This interaction may initiate subsequent reactions such as proteolytic cleavage and release (from inhibition) of the central DNA binding domain (Molinari *et al*., 1996; Okorokov *et al*., 1997), assembly of repair proteins (Lee *et al*., 1995), and induced accumulation of p53 due to protein stabilization (Maltzman and Czyzyk, 1984).

We are concerned here with the question of how the stability of the protein is enhanced by treatment of cells with DNA damaging agents. Induced stabilization depends on damage introduced into the transcribed part of the genome (Yamaizumi and Sugano, 1994). In non-treated cells p53 appears to be degraded through the ubiquitin-dependent 26 S proteasome pathway (Scheffner *et al*., 1993; Maki *et al*., 1996). Blocking of the pathway by specific peptide aldehydes leads to accumulation of p53 *in vivo* and ubiquitin-p53 conjugates are detectable in both non-treated and gamma-irradiated, but not in UV-irradiated, human cells (Maki *et al*., 1996; Maki and Howley, 1997).

In cotransfection experiments the product of the mdm2 gene which is transcriptionally induced by p53, mediates the degradation of exogenously expressed p53 and this Mdm2-induced destabilization of p53 depends on the physical interaction of the two proteins (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Böttger *et al*., 1997). Cells expressing a DNA-binding defective mutant of p53 accumulated p53 because of their inability to induce Mdm2 expression (Midgley and Lane, 1997). Mdm2-dependent p53 degradation proceeds through the proteasome pathway (Kubbutat *et al*., 1997) and experiments with purified components have suggested that Mdm2 functions as a p53 specific ubiquitin ligase of the E3 type (Honda *et al*., 1997).

It is not yet known what determines the stability of p53 under various conditions and how its stability is increased in irradiated cells. One possibility is that Mdm2 may be inactivated in irradiated cells. Alternatively the accessibility of p53 to the ubiquitin system may be regulated. Accessibility is likely to depend on conformational properties which could be influenced by interaction with another protein (e.g. SV40 large T antigen) or with DNA (e.g. the DNA damage site), or by posttranslational covalent modification (e.g. phosphorylation) triggered by the transcriptional arrest. The possibility of p53 turnover regulation by phosphorylation is supported by several arguments: p53 is indeed heavily phosphorylated and is the putative target for numerous protein kinases (reviewed in Meek, 1994). Phosphorylations and dephosphorylations may profoundly affect protein conforma-

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tion. A host of protein kinases are activated in irradiated cells (reviewed in Bender *et al*., 1997). In fact, gamma irradiation promotes phosphorylation of serine 15 of the human p53 protein, and the phosphorylation of this serine and of serine 37 appears to interfere with the association of p53 with Mdm2 (Shieh *et al*., 1997).

In a systematic study of p53 mutations in which we searched for the domain of p53 required for DNA damage induced stabilization, we found that the carboxyterminal 30 amino acids and several putative phosphorylation sites, singly or in combination, can be mutated without effect on induced stabilization. The aminoterminal serine substrates which are putatively addressed by casein kinase I, the JNK target serine 33, serines 315 (Cdk2) and 392 (casein kinase II), and one of the Erk phosphorylation sites (which are not conserved between man and mouse) do not mediate stabilization. Also a double mutation of serines 15 and 37, which may be targeted by an as yet unknown member of the DNA-PK family, does not appear to interfere with UV or actinomycin D induced p53 stabilization. Several mutants code for stable p53 proteins, e.g. F19S/L14Q which does not interact with Mdm2, as well as mutants in the oligomerization domain or in the nuclear localization domain. These cannot be further stabilized in response to DNA damage. p53 turnover occurs obviously in the nucleus and Mdm2 dependent destabilization is needed for subsequent stabilization. Overexpression of Mdm2, however, inhibits UV-induced p53 stabilization suggesting that stabilization depends critically on the p53/Mdm2 ratio, and also indicating that p53 in UV irradiated cells is still an Mdm2 substrate.

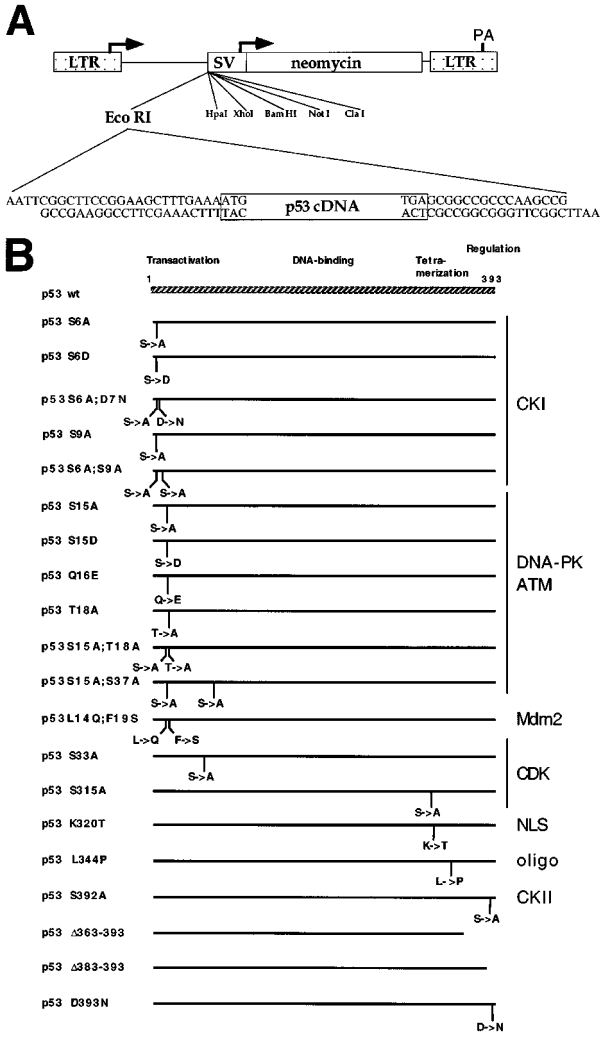

Figure 1 Retroviral constructs encoding p53. (**a**) p53 wild type construct in the vector pLXSN, showing the non-p53 sequences surrounding the p53 open reading frame, the cloning sites and the position of the neomycin gene. LTR: long terminal repeat of Mo-MuSV and Mo-MuLV respectively; SV: simian 40 virus enhancer; PA: polyadenylation site; the arrows indicate transcriptional start sites. (**b**) p53 wild type showing the functional domains of the protein, and p53 mutants used in this study. On the right side the enzymes reportedly phosphorylating the serines/threonines that had been mutated, or involvement of the mutated amino acids in p53 function are indicated: CKI: casein kinase I; DNA-PK: DNA dependent protein kinase; ATM: protein kinase deficient in Ataxia teleangiectasia; Mdm2: residues involved in binding of Mdm2; CDK: cyclin dependent kinase; NLS: nuclear localization signal; oligo: amino acid involved in the oligomerization of the protein; CKII: casein kinase II

Results and discussion

The experimental systems

In order to test mutated p53 for its stability and inducible stabilization, a cell system is required that coregulates exogenous p53 (expressed from a transfected expression plasmid) and endogenous p53 protein. We use here, partly in parallel, two systems to investigate the expression of human wild type and mutant p53 in mouse cells: (i) we generated stable transfectants in the murine retroviral packaging cell line ΩE (Morgenstern and Land, 1990) and (ii) we developed a transient transfection system in which human p53 was transiently expressed in either ΩE cells, NIH3T3 fibroblasts or fibroblasts derived from $p53^{-/-}$ embryos. Both stable and transient transfections yielded essentially identical results in the analysis of p53 mutants (the expression construct and a survey of the mutants tested are shown in Figure 1).

Stably expressed wild type p53 is regulated in ΩE cells identically to the murine p53

In the murine retroviral packaging cell line ΩE the endogenous murine p53 protein is stabilized by treatment of the cells with UV light, γ rays or actinomycin D (Figure 2a, upper panel), demonstrating that the pathways leading to p53 stabilization are intact in this cell line. The experiment further suggests that ΩE cells express wild type murine p53. Assays of reporter genes activated by p53 also support the notion that p53 is not mutated in ΩE cells (not shown).

We stably expressed in ΩE cells wild type or mutant forms of human p53 under the control of a retroviral promoter (Figure 1). The human protein can be distinguished from murine p53 by its larger size (Figure 2a, lower panel) and through the use of antibodies specific for human p53 (Figure 2b). Wild type exogenous human p53 is indeed stabilized in these cells after irradiation with UV of γ, or after treatment with actinomycin D, similarly to the endogenous murine protein (Figure 2a, lower panel, and Figure 2b). All

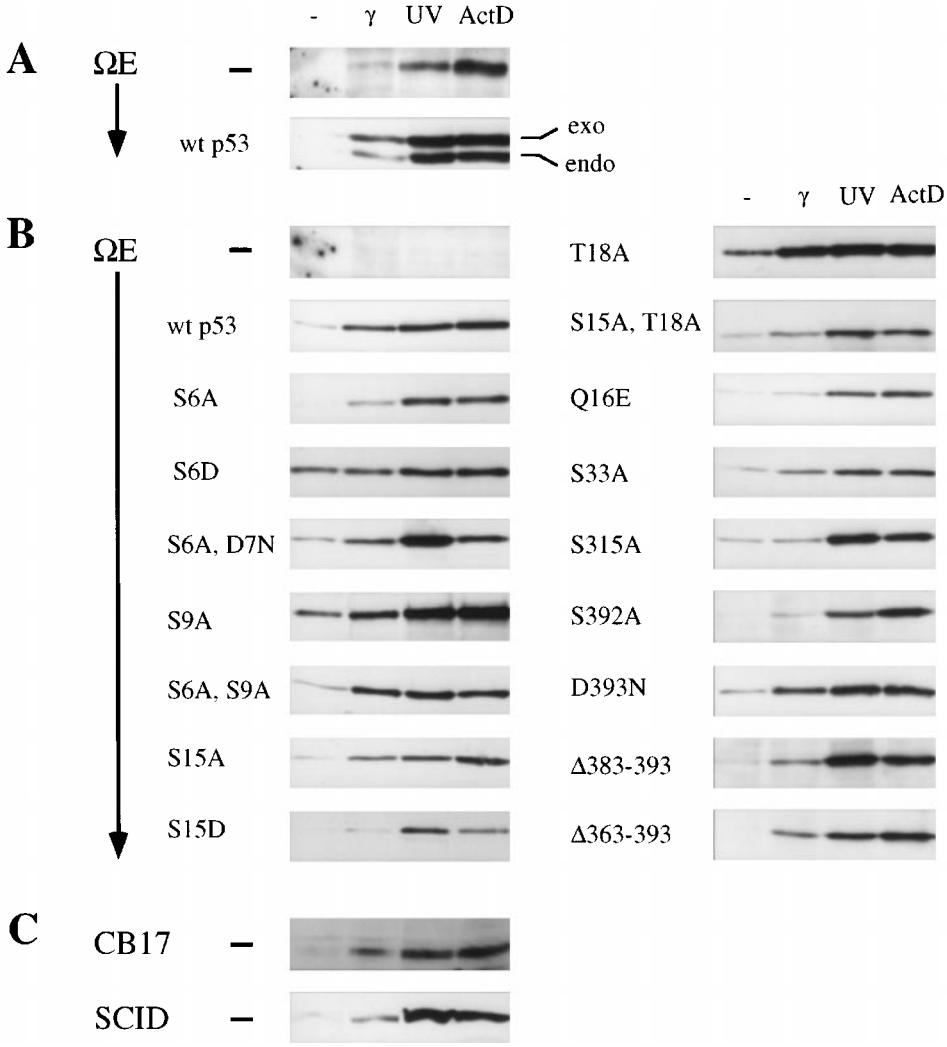

Figure 2 Inducible stabilization of wild type and mutant p53 proteins in stable transfectants and in fibroblasts from DNA-PK negative scid mice. (**a** and **b**). ΩE cells and ΩE cells stably transfected with p53 wild type or various p53 mutants were treated with γ-rays (5 Gy), UVC (30 J/m^2) or actinomycin D (Act D, 2.5 μg/ml). Nuclear extracts were prepared at 1 h (γ-rays) or 4 h (UVC, Act D) after treatment and proteins (70 μg per lane) were resolved by SDS–PAGE. The proteins were transferred to nitrocellulose membrane and probed with (**a**) Pab 421, recognizing both human and murine p53, or (**b**) DO-1, specific for human p53. The Western blots were developed by ECL. Note that expression levels between transfectants differ. Although normalized for the endogenous p53 response, comparisons should only be made within each panel. Only induction factors can be compared between panels. (**c**) Basal and induced p53 levels in murine skin fibroblasts from CB17 and scid mice. Primary fibroblasts were treated as above and analysed for p53 expression with the monoclonal antibody Pab122

experiments with transfectants were evaluated for both the exogenous and the endogenous p53 proteins (only shown in Figure 2a). Basal level and induction of endogenous p53 served to confirm successful treatment with radiation or actinomycin D and to make sure that the loading of the gel was correct.

To study posttranslational processes, the LTR construct should not be inducible transcriptionally. Indeed, there is no detectable transcriptional activation of the viral LTR controlling the expression of p53 as shown by Northern analysis. The probe detects two endogenous transcripts (Figure 3), only the larger of which comigrates with p53 RNA detectable in 3T3 cells (not shown), and a larger human p53-neo transcript (Figure 3; see also the expression construct in Figure 1a). None of the inducers enhanced the abundance of endogenous or exogenous p53 RNA (Figure 3) confirming earlier reports (Maltzman and Czyzyk, 1984; Lu and Lane, 1993; Fritsche *et al*., 1993) and demonstrating that in ΩE cells the viral LTR promoter directing the transcription of human p53 is not activated by UV light, γ rays or actinomycin D.

To directly verify posttranslational stabilization of exogenous p53, we determined the half life of p53 protein in pulse-chase experiments. The half life of exogenous wild type p53 was 25 min in non-treated stably transfected ΩE cells (Figure 4). Upon treatment with UV or actinomycin D, p53 became stable. No decrease in p53 levels within the chase period of 60 min could be detected (quantitation in Figure 4b). Mutant proteins to be described below, showed the same half life as wild type p53 (exemplified for Q16E and D393N in Figure 4c).

In agreement with a previous report (Lu and Lane, 1993), the time course of p53 stabilization differed with the inducer used: γ rays stabilized p53 readily but transiently with maximal levels at 120 min (Figure 5a). Actinomycin D induction was also rapid, but maximal

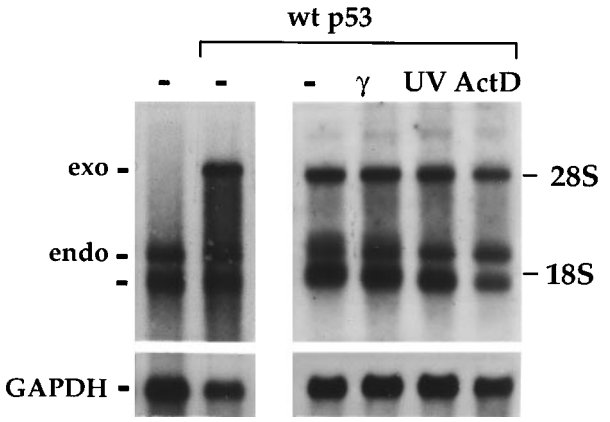

Figure 3 No induction of exogenous or endogenous p53 RNA synthesis by γ or UVC irradiation or by actinomycin D treatment. Poly(A)$^+$ mRNAs were prepared and 2 μg each were resolved on 1.4% formaldehyde-agarose. After transfer to nitrocellulose, the membrane was probed consecutively with ^{32}P-labelled mouse p53 and GAPDH cDNAs, and exposed to film to roughly same band intensity. RNA loaded was from ΩE cells (left-most lane), or ΩE cells stably transfected with p53 wild type (lanes 2–6). Lanes 1–3: non-treated. Lanes 4–6: treatments as in Figure 2. The probe hybridizes to two endogenous RNA species the larger of which corresponds to the expected p53 RNA size. We do not know whether the smaller band represents a splice variant or a mutated allele. The exogenous RNA species comigrates with 28 S RNA and represents a chimeric p53-neo RNA which also hybridizes with a neomycin probe. Exogenous RNA is absent from lane 1. The bicistronic RNA has no influence on p53 expression. Human and mouse hybridization probes yielded identical results (not shown)

levels were maintained and increased even further for more than 24 h. Interestingly UV caused p53 stabilization with a lag period of 120 min, and the stabilization was as long-lasting as that induced by actinomycin D treatment. In most subsequent experiments p53 abundance was determined at 1 h after γ irradiation and at 4 h after UV or actinomycin D treatment.

Thus we can conclude that human p53 accumulation in stably transfected murine cells can be enhanced to the same extent as that of the endogenous protein by a variety of agents. The enhancement is due to posttranslational stabilization. The molecular requirements for stabilization of p53 are apparently conserved between man and mouse, and the protein-coding region of p53, the only part present in the transfected gene construct, suffices for induced stabilization of the protein. Untranslated RNA sequences which possibly control translation of the protein (Mosner *et al*., 1995) are not contained in the construct. Most importantly, the cell system is suitable for the analysis of p53 mutants.

Single putative phosphorylation sites and the 30 carboxyterminal amino acids are not involved in DNA damage or transcriptional arrest induced p53 stabilization

Our mutant screen was intended to explore one of the most plausible posttranslational modifications to be involved in protein stability, phosphorylation. p53 is in fact heavily phosphorylated (reviewed in Meek, 1994) and changes in phosphorylation could well be the basis of altered stability. Most mutants, however, upon stable transfection into ΩE cells, showed no difference in induced stabilization. Examples of this screen are shown in Figures 1b and 2b.

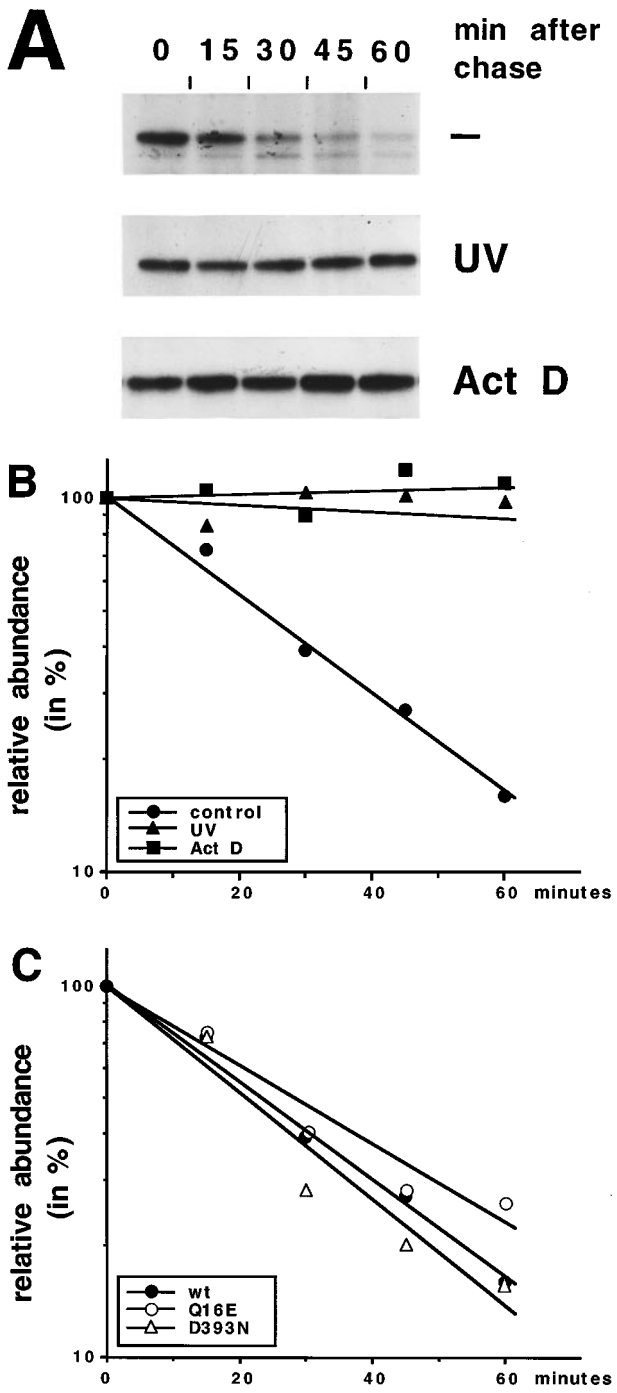

Figure 4 Half life of p53 proteins determined by pulse-chase experiments. (**a**) ΩE cells, stably transfected with wild type p53 were labelled with L-^{35}S-methionine and L-^{35}S-cysteine and treated with UVC (30 J/m^2) or actinomycin D (2.5 μg/ml) or not treated for 5 h. The culture medium was then replaced by non-radioactive culture medium supplemented with ten times the normal concentration of methionine and cysteine. The cells were harvested at the time points indicated, p53 was immunoprecipitated with the monoclonal antibody DO-1 and the p53-antibody complexes were resolved by SDS–PAGE. An autoradiogram is shown. (**b**) The time course of p53 degradation in non-treated, UVC or actinomycin D treated cells. Quantitation of data as shown in panel a was performed by scanning the X-ray films with a densitometer (LKB). (**c**) Time course of spontaneous degradation of the p53 mutant proteins D393N and Q16E in comparison to wild type p53 in ΩE cells stably expressing the appropriate constructs

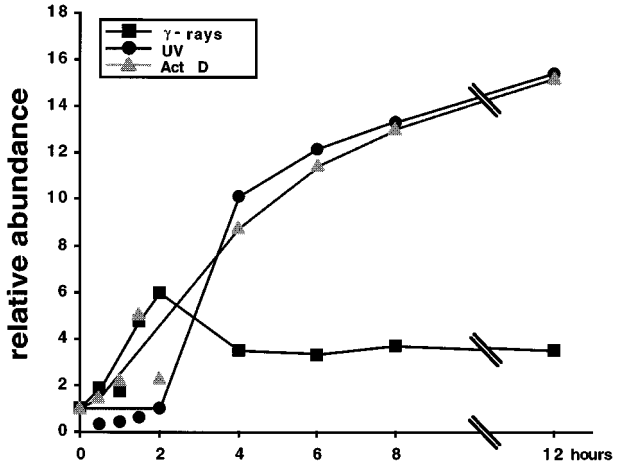

Figure 5 Time course of p53 stabilization. ΩE cells stably transfected with wild type p53 were irradiated with γ or UVC or treated with actinomycin D as indicated. At various time points cells were harvested and analysed for the level of human p53 by Western blot analysis with the antibody DO-1. ECL exposures were scanned with a densitometer and the level of p53 expression at the time of treatment was set 1

For instance, the putative Erk 1,2 recognition site, serine 73 (Milne *et al*., 1994), is not involved in protein stability, as it does not exist in human p53.

Serines 4, 6 and 9 of the murine p53 are phosphorylated *in vivo* and *in vitro* by a casein kinase I like enzyme (Milne *et al*., 1992) with properties indistinguishable from casein kinase I δ and I ε. Moreover, treatment of cells with topoisomerase-inhibiting drugs increased casein kinase I in a p53 dependent manner suggesting an autoregulatory loop (Knippschild *et al*., 1997). Mutation of these serines, singly or in combination (S6A; S6D, S9A, S6AD7N, S6AS9A), did not alter the induced stabilization of the mutant proteins. This suggests that phosphorylation of these sites is not involved in the immediate reaction of p53 to DNA damaging agents (Figure 2b).

Mutant Q16E destroys the recognition site for DNA dependent protein kinase (DNA-PK; Lees-Miller *et al*., 1992), and the serine 15 to alanine or to aspartic acid mutation destroys one of the acceptor sites for DNA-PK (an additional substrate site in the human protein is serine 37). Threonine 18 appears to be phosphorylated *in vitro* by the protein kinase defective in Ataxia teleangiectasia (ATM). Several mutant proteins affecting the recognition by either DNA-PK or ATM expressed in several independent cell clones, were as stable as the wild type protein in non-treated cells, and were further stabilized upon irradiation with either γ or UV, or upon treatment with actinomycin D (Figure 2b). The results are consistent with the finding that in primary fibroblasts or thymocytes from 'scid' mice which lack DNA-PK activity (Blunt *et al*., 1995), p53 was induced by γ, UV and actinomycin D just as in cells derived from wild type mice (CB 17, Figure 2c, and Gurley and Kemp, 1996; Fried *et al*., 1996). In agreement with this result, p53 dependent cell cycle arrest and apoptosis are preserved in 'scid' fibroblasts (Huang *et al*., 1996; Candeias *et al*., 1997) and ionizing radiation induces p53 accumulation in 'scid' thymocytes (Nacht *et al*., 1996).

Serine 33 is the putative site for JNK dependent phosphorylation and a major substrate for cdk7-cyclinH-p36^{mat1} (the cyclin-dependent kinase activating kinase, CAK) *in vitro* and *in vivo* (Ko *et al*., 1997; others, however, have mapped the CAK-dependent phosphorylation sites to residues between positions 311 and 392, Lu *et al*., 1997). CAK is part of the transcription factor II H multiprotein complex which is required for RNA polymerase dependent transcription and nucleotide excision repair (Svejstrup *et al*., 1996; Hoeijmakers *et al*., 1996). Mutating serine 33 to alanine did not affect DNA damage or actinomycin D induced stabilization of p53 (Figure 2b).

Other cyclin-dependent kinases phosphorylate human p53 at position 315 (Bischoff *et al*., 1990; Wagner *et al*., 1998). Also mutation of this phosphorylation site did not affect induced stabilization (Figure 2b).

Finally, we wish to report on mutations in the C-terminal domain of p53. Serine/threonine residues in this region are targeted by casein kinase II (position 392) and by PKC *in vitro* and *in vivo* (Meek *et al*., 1990; Baudier *et al*., 1992; Hupp and Lane, 1994; Milne *et al*., 1996; Youmell *et al*., 1998). These phosphorylations upregulate the DNA binding function of the central p53 domain *in vitro* (Hupp *et al*., 1992; Hupp and Lane, 1994; Takenaka *et al*., 1995) and phosphorylation of serine 392 stabilizes the homotetramer formation of p53 (Sakaguchi *et al*., 1997). The C-terminal domain itself has been reported to bind to single-stranded DNA (Lee *et al*., 1995; Jayaraman and Prives, 1995). The C-terminus is therefore of particular interest as a target for induced structural changes. Neither mutation generated (Figure 1b) did affect spontaneous or induced stability of p53: attachment of a His-tag to the C-terminal aspartic acid residue (not shown); mutation of the last amino acid (D393N) (Figures 2b and 4c); deletion of C-terminal amino acids (p53Δ383-393 and p53Δ363-393); amino acid exchange at position 392 (S392A) (Figure 2b).

To our knowledge, only two previous reports have examined p53 with altered C-terminal ends. Hupp and Lane (1995) expressed a human p53 protein lacking the four most carboxy-terminal amino acids in insect cells and demonstrated that this mutated protein behaved similarly to the wild type protein with respect to specific DNA and antibody binding. Second, a shorter alternatively spliced form of mouse p53 exists naturally where the 26 C-terminal amino acids of the predominant splice form are replaced by a different sequence of 17 amino acids. The alternatively spliced p53 protein binds constitutively to p53 response elements, but does not participate in molecular interactions known to occur between the C-terminal end of full-length p53 and other cellular molecules (Wu *et al*., 1994; Bayle *et al*., 1995). The protein seems to be less abundant than normal p53 in certain murine cell lines and less inducible by actinomycin D (Kulesz-Martin *et al*., 1994). These features were not seen with the deletion mutants generated in this study.

In summary, we have demonstrated here that mutation of all phosphorylation sites examined, singly or in selected combinations, or the deletion of the 30 carboxyterminal amino acids do not affect the properties of p53 analysed here, namely low level expression in non-treated cells and stabilization in cells irradiated with UV or γ, or in cells treated with actinomycin D.

Our findings also indicate that the reported molecular interactions of the C-terminus of p53, e.g. with damaged DNA (Jayaraman and Prives, 1995; Bayle *et al*., 1995), 5.8 S ribosomal RNA (Fontoura *et al*., 1992), casein kinase II (Herrmann *et al*., 1991), with members of the TFIIH complex (Wang *et al*., 1995) or single-strand binding protein RPA (Dutta *et al*., 1993), are irrelevant for DNA damage induced stabilization.

Analysis of p53 stabilization in transient transfections

Our findings suggest that all mutant proteins tested so far and stably expressed in ΩE cells are stabilized by DNA damaging agents. As one caveat for this unexpected result we need to consider whether the endogenous murine wild type p53 protein could, through heterooligomerization, costabilize the exogenous human protein such that we could not detect the influence of the mutations on stability. Two arguments speak against this interpretation. (I) By chance we cloned p53 in the inverse orientation into the LTR vector. This gave rise to very low protein levels of p53 in the stably transfected cells, because of the low activity of the 3′LTR. Although p53 at this low level was stabilized by UV or γ irradiation by the same factor as was correctly cloned p53 (with the higher basal level of expression), the low p53 level was barely enhanced by treatment of the cells with actinomycin D (results not shown). This finding is not compatible with the idea of heterooligomerization and costabilization of the human and murine protein. (ii) Analyses of the influence of phosphorylation on the oligomerization state of p53 suggest that in normal undamaged cells p53 may be largely monomeric (Sakaguchi *et al*., 1997). In order to prove that our results were not caused by oligomerization of mutant human p53 with wild type mouse p53, we designed an independent approach. Because we did not succeed in establishing stable transfectants of human p53 in mouse $p53^{-/-}$ fibroblasts, we developed a transient transfection system which allowed for the stabilization of the transiently expressed p53 proteins. Quantitively similar results were obtained with ΩE cells, NIH3T3 cells, and with $p53^{-/-}$ cells, excluding heterooligomerization as a cause of stabilization.

Transient transfection of p53 driven by the viral LTR, into ΩE cells (Figure 6a, left panel), NIH3T3 cells (Figure 6a, upper right panel) or into $p53^{-/-}$ fibroblasts (Figure 6a, lower right panel) with the Ca-phosphate precipitation method gave rise to low levels of p53 protein which were moderately enhanced by γ irradiation (shown in Figure 6 for $p53^{-/-}$ cells, and in Figure 7a for NIH3T3 cells) and strongly by UV irradiation or by treatment of the cells with actinomycin D (Figures 6 and 7). However, induction factors did not reach the same levels as those of the endogenous protein. Lowering the amount of transfected p53 plasmid and prolonging the time interval between transfection and treatment of the cells did not increase stabilization factors.

As discussed for stable transfection experiments, the DNA damage induced increase in p53 level is due to the stabilization of the protein and not to enhanced synthesis. (i) The LTR driving the p53 gene is not transcriptionally induced by radiation (Figure 3) and actinomycin D, at the concentrations used (2.5 μg/ml),

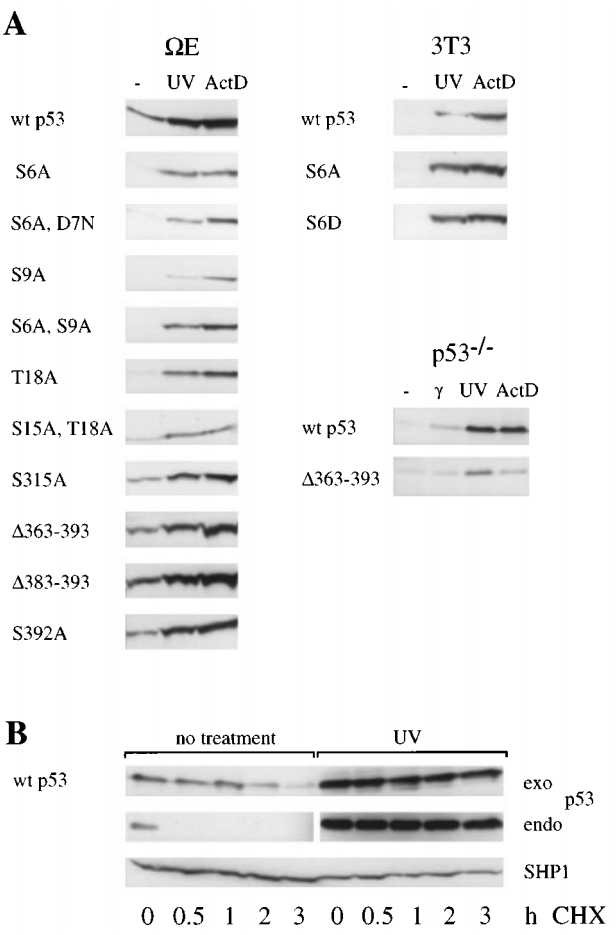

Figure 6 DNA damage induced stabilization of p53 expressed from transiently transfected plasmids. (**a**) ΩE cells, NIH3T3 fibroblasts, and $p53^{-/-}$ fibroblasts were transiently transfected by calcium phosphate precipitation with plasmids at indicated. Twenty-four hours after transfection cells were treated as indicated and as described in Figure 2, and harvested at 1 h (γ-irradiation) or at 6 h (UV irradiation, actinomycin D treatment) after treatment. Cell extracts were resolved by SDS–PAGE, and Western blots were performed with the antibody DO-1. (**b**) UV-induced stabilization of wild type p53 expressed from a plasmid transiently transfected into NIH3T3 fibroblasts. Twenty-four hours after transfection, cells were irradiated with UV. After 6 h, cycloheximide (CHX) was added to the cultures. The cells were harvested at the indicated time points and 150 μg of the cell extracts from non-treated cells and 50 μg cell extracts from the UV irradiated cells were resolved by SDS–PAGE. Western blots with the DO-1 antibody were performed as in (**a**). The gel was reprobed with Pab 421 for endogenous p53 and an antibody specific for the tyrosine phosphatase SHP1. Note that the gel showing endogenous p53 required five times longer exposure. Also in transient transfections, direct comparisons of absolute expression levels between panels may not be informative as plasmids and transfection efficiencies may differ

inhibits p53 transcription (not shown). (ii) The level of several stable p53 proteins is not further increased by irradiation or by actinomycin D (see below). In an attempt to directly measure half-life, we first permitted p53 accumulation for 24 h after transfection, then treated with cycloheximide in order to inhibit new p53 synthesis, and followed p53 degradation in the subsequent 3 h. While in non-irradiated cells, exogenous p53 disappeared with a half-life of about 3 h, it was stable when cells had been irradiated with UV 6 h prior to the addition of cycloheximide (Figure 6b). The endogenous murine p53 behaved accordingly, with the

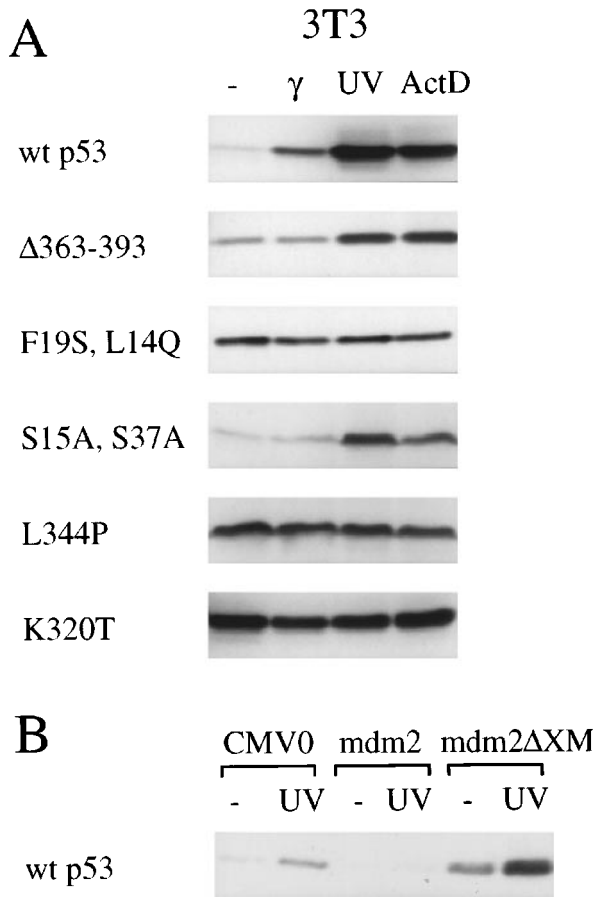

Figure 7 Mdm2-mediated degradation of p53 as a prerequisite of induced p53 stabilization. (**a**) The indicated p53-encoding plasmids were transiently transfected into NIH3T3 cells. Twenty-four hours after transfection the cells were not treated (−), γ-irradiated, UV-irradiated or treated with actinomycin D as in Figure 2. One hour (γ-irradiation) or 6 h (UV, actinomycin D) after treatment, cell extracts were prepared and probed in Western blots with the antibody Pab 1801 for the level of human p53. (**b**) 5 μg human wild type p53 expression clone were cotransfected with 10 μg CMV O (empty vector), 10 μg CMV plasmid driving murine mdm2 cDNA, or 10 μg CMV driving murine mdm2 cDNA lacking the p53 interaction domain (mdm2ΔXM, Barak *et al*., 1994). Twenty-four hours after transfection, cells were irradiated with UV or not irradiated and harvested after 6 h. Cell extracts were analysed for the level of human p53 with the DO-1 antibody as in **a**

difference that in non-treated cells it disappeared with a half-life of 15 min. Thus, in these transient transfection conditions, exogenous p53 is spontaneously more stable than p53 in non-transfected cells (Renzing and Lane, 1995) and nevertheless, can be further stabilized. This difference may be explained either by assuming that a stabilization signal is generated by the DNA uptake during transient transfection, or that p53 in vast excess as synthesized in transiently transfected cells slows down turnover. Most importantly, the experiment shows that human p53 after transient transfection, can be further stabilized by UV irradiation and that p53 mutants can be analysed in transient transfections.

Figure 6a shows the results for several p53 mutants. All mutants in putative phosphorylation sites and the carboxyterminal deletion mutants which had been analysed in stable transfectants, were also stabilized by UV or actinomycin D in transient transfections. Analysis for γ-induced stabilization in transient transfections was not conclusive, because γ-induced stabilization was low and variable. With the carboxy-terminal deletion mutant Δ363-393 (Figures 6a and 7a) and with the double mutant (S15A;S37A) (Figure 7a) we did not see γ-induced stabilization, but we do not know whether this was due to an enhanced basal stability of the protein (as described previously, Meek, 1994; Midgley and Lane, 1997), or to the specific involvement of the mutated amino acids. Importantly, combined mutation of the two serines targeted by DNA-PK did not prevent UV and actinomycin D induced stabilization of p53 (Figure 7a, S15A;S37A).

Requirement for Mdm2

Upmodulation of p53 stability can only be achieved if the spontaneous turnover is high. This is, as introduced above, the function of the p53 partner protein Mdm2. Several p53 mutant proteins are spontaneously stable, e.g. that encoded by mutation F19S/L14Q (Figure 7a) which prevents the interaction with Mdm2 (Lin *et al*., 1994). Their protein level is not changed by DNA damaging agents (nor by treatment of the cells with the proteasome inhibitor MG132, which stabilizes the wild-type p53 protein). Interestingly, the F19S/L14Q mutation destroys the epitope for the antibody DO-1 (not shown) which upon intracellular expression interferes with stability regulation of p53 (Cohen *et al*., 1998). Also several mutations in the p53 oligomerization domain (Stürzbecher *et al*., 1992; Ishioka *et al*., 1995) led to the synthesis, after transient transfection, of apparently stable proteins whose levels were not further enhanced by radiation or actinomycin D (F341K; L344E and L348E;A353K (not shown) and L344P (Figure 7a)). Oligomerization of p53 appears to be a prerequisite for Mdm2 binding (Marston *et al*., 1995), also compatible with a requirement for Mdm2 in the regulation of p53 stability. Consistent with this, antisense inhibition of Mdm2 synthesis enhanced functional p53 levels and upregulation by chemotherapeutic agents (Chen *et al*., 1998).

p53 is a nuclear protein and its turnover should be regulated in the nucleus. We wondered what would happen if we accumulated p53 in the cytoplasm. The mutation K320T which destroys the most important nuclear localization signal of human p53 accumulation, encodes a protein which could not be further enhanced by irradiation or actinomycin D (Figure 7a). Identical results were obtained in $p53^{-/-}$ cells (results not shown). These data are reminiscent of the high accumulation of wild type p53 in the cytoplasm of certain neuroblastoma cell lines (Moll *et al*., 1995). Possibly there is no Mdm2 in the cytoplasm or cytoplasmic proteins interfere with Mdm2 binding to p53.

Excess Mdm2 prevents induced p53 stabilization

As shown above, Mdm2-induced destabilization of p53 is a prerequisite for subsequent DNA damage induced stabilization. Should elevated levels of Mdm2 then not further improve inducibility? This is, however, not the case: overexpression of murine Mdm2, but not of Mdm2 lacking its interaction domain with p53 (Barak *et al*., 1994), inhibited UV induced stabilization of p53 (transient coexpression; Figure 7b). These data suggest

that the process of DNA damage induced p53 stabilization depends on a carefully adjusted level of active Mdm2. Possibly proteins of the Mdm2 pathway are the primary targets of posttranslational signalling triggered by arrested transcription.

Conclusions

Our experiments demonstrate that the only single requirement for p53 regulation by DNA damaging agents is its prior destabilization by the protein Mdm2. Neither single putative phosphorylation sites nor interactions of the C-terminal part of the protein with cellular macromolecules mediate radiation or actinomycin D induced stabilization. The possibility remains that stabilization proceeds in a redundant fashion, so that several functions of the p53 protein have to be destroyed in order to prevent DNA damage induced stabilization. Alternatively DNA damage induced stabilization may be triggered by a (e.g. inactivating) modification of Mdm2 or of proteins controlling Mdm2 levels (Zhang *et al*., 1998; Pomerantz *et al*., 1998).

Materials and methods

Cloning of p53 and mutagenesis

p53 was amplified by PCR using the following primers: 5′-CCG GAAGCTTT GAAAAT GGAGGAGCCGCAGTCA-GATCCT-3′ and 5′-GGGCGGCCGCTCAGTCTGAGT-CAGGCCCTTCTG-3′ and 30 cycles of: 1 min at 94°C, 1 min at 50°C and 3 min at 72°C, followed by a 10 min extension period at 72°C at the end of the PCR reaction. Reverse-transcribed RNA from EBV-transformed human lymphoid cells expressing wild type p53 served as template. The PCR reaction was carried out under standard salt conditions using a reaction buffer supplied by the manufacturer of the Taq polymerase (Amersham). The amplified fragment was ligated into the pCR™II vector (Invitrogen). p53 sequences were excised by *Eco*RI and ligated into the retroviral vector pLXSN (Miller and Rosman, 1989). The p53 coding region was sequenced in both directions and found to be identical to the published human sequence (Zakut-Houri *et al*., 1985). Site-directed mutations were introduced by PCR, using p53 wild type cDNA as template and primers covering the desired mutations. These PCR products resulted in full-length mutant p53 products containing the desired mutations. The PCR products were cloned into the pCR™II vector, excised and ligated into the retroviral vector PLXSN as described above. The cDNAs were sequenced to verify the mutations introduced and found to have no additional mutations.

Cell lines and their treatment

ΩE cells were obtained from Hartmut Land, London; $p53^{-/-}$ mouse embryo fibroblasts ($p53^{-/-}$ MEF; Donehower *et al*., 1992) were obtained from Michael Fritsche, Freiburg. Primary skin fibroblasts were grown out (2 weeks) from the skin of newborn 'scid' mice (obtained from Charles River, Sulzfeld, Germany) or CB 17 wild type mice (obtained from Piet Nielson, Freiburg). NIH3T3 cells were obtained from Peter Gruss, Göttingen. All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in humidified 6% CO_2. Cells were irradiated in culture medium with 5 Gy in a cobalt-60 γ-source at a dose rate of 5.75 Gy/min. Prior to UVC irradiation, medium was removed and the cells were washed once with PBS. The cell layer was then irradiated with a germicidal lamp (254 nm, 30 J/m^2) and further cultured in the original conditioned medium. Actinomycin D-mannitol (Sigma) dissolved in water was added to the cultures at a final concentration of 2.5 μg/ml. Cycloheximide was added at 10 μg/ml.

Pulse chase

Cells were grown in DMEM without methionine and cysteine, supplemented with 10% dialysed FCS and 150 μCi/ml Pro-mix™ (Amersham; containing L-[^{35}S] methionine and L-[^{35}S]cysteine) for 5 h. The medium was then removed and the cells were washed extensively with complete medium and further incubated in complete medium plus a tenfold higher concentration of non-radioactive methionine and cysteine.

Transfections

ΩE cells, NIH3T3 and $p53^{-/-}$ MEF cells were transfected by calcium phosphate-DNA coprecipitation (Chen and Okayama, 1987). Cells were treated 24 h after transient transfection and harvested at the indicated time points. For stable transfectants, ΩE cells were selected for G418 resistance (800 μg G418/ml). Expression clones for Mdm2: pCOCmdm2x2 (wild type mdm2 from mouse); pCOCmdm2ΔXM (mouse; deletion of the p53 binding domain; see Midgley and Lane, 1997).

RNA preparation, Northern blots, cDNA probes

Poly (A)$^+$-RNA was isolated according to Rahmsdorf *et al*. (1987) and resolved on a 1.4% agarose-formaldehyde gel, transferred onto a Hybond N$^+$ nylon membrane and hybridized with an *Eco*RI fragment of mouse p53 cDNA spanning the whole open reading frame. Filters were reprobed with a *Pst*I fragment of the open reading frame of mouse glyceraldehyde-3-phoshate-dehydrogenase (GAPDH, provided by Dr P Curtis, Philadelphia).

Preparation of cell extracts, SDS–PAGE, Western blotting

Cells were washed twice in ice cold PBS, scraped into PBS with a rubber policeman and centrifuged at 1000 *g* for 5 min. For nuclear extracts, the cells were disrupted in ice-cold 10 mM HEPES pH 7.9; 60 mM KCl; 1 mM EDTA; 0.5% NP-40; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were sedimented at 800 g for 5 min and were washed once in the same ice-cold buffer but omitting NP-40. Nuclei were resuspended in ice-cold 250 mM Tris pH 7.8; 60 mM KCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF, frozen and thawed three times and centrifuged at 13 000 *g* for 10 min. For whole cell extracts, cells were disrupted in 250 mM Tris pH 7.8; 60 mM KCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF, frozen and thawed three times and centrifuged at 13 000 *g* for 10 min. Protein concentration of the supernatant (protein extract) was determined according to Bradford (Bio-Rad). Western blots were performed as described previously (Blattner *et al*., 1994) and were probed with the following antibodies: Pab 421 (recognizing human and mouse p53, Oncogene Science), DO-1 (recognizing only human p53, Santa Cruz), Pab 122 (recognizing human and mouse p53, Boehringer, Mannheim), and Pab 1801 (human p53, Santa Cruz).

Immunoprecipitation

Cells were washed twice in ice-cold PBS and lysed in ice-cold RIPA-buffer (50 mM Tris/HCl, pH 8.0; 125 mM NaCl;

0.5% IGEPAL CA-630; 0.5% sodium desoxycholate; 0.1% sodium lauryl sulfate; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 mM PMSF). The lysate was centrifuged at 150 000 *g* and 4°C for 20 min. The supernatant was added to 5 μg DO-1 antibody precoupled to protein G plus agarose (Calbiochem) and incubated on a rocking platform at 4°C for 90 min. The agarose-antibody-p53 complexes were collected by centrifugation, washed four times in RIPA-buffer, resuspended in sample buffer and resolved by SDS–PAGE (Blattner *et al*., 1994). The gels were fixed in 30% methanol/10% acetic acid for 20 min, washed in water for 20 min and enhanced in 1 M sodium salicylate/30% methanol for 20 min. The gels were dried and exposed to X-ray film.

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