

Induced N- and C-terminal cleavage of p53: a core fragment of p53, generated by interaction with damaged DNA, promotes cleavage of the N-terminus of full-length p53, whereas ssDNA induces C-terminal cleavage of p53

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p53 is able to recognize and bind sites of DNA damage and, in some way, damage to cellular DNA activates a p53 response leading to G₁ arrest or apoptosis. We have previously shown that ‘damaged DNA’ induces N-terminal cleavage of p53 to generate p40(ΔN) and p35 (core) protein products. We now show that the p35 product has protease activity and is able to cleave between residues 23 and 24 of full-length p53 to generate a novel product, p50(ΔN23). This activity was inhibited by bestatin, an aminopeptidase inhibitor. Residues 23 and 24 lie within the mdm-2 binding domain of p53 and the possibility that p50(ΔN23) may be resistant to feedback regulation by mdm-2 is discussed. Unexpectedly, interaction with ssDNA induced two further cleavage products of p53, generated by C-terminal cleavage and designated p50(ΔC) and p40(ΔC). *In vivo* generation of a C-terminal cleavage product of endogenous p53 similar in size to p50(ΔC) correlated with up-regulation of p21 expression in ML-1 cells exposed to either adriamycin or cisplatin. The possible significance of the various p53 cleavage products in relation to the cellular response to DNA damage is discussed.

Keywords: activation/autoproteolysis/DNA damage/mdm-2/p53

Introduction

The p53 tumour suppressor is able to induce growth arrest or apoptosis in DNA-damaged cells and in this way p53 protects against genetic instability and predisposition to cancer (for recent reviews see Gottlieb and Oren, 1996; Kastan, 1996; Ko and Prives, 1996; Levine, 1997). Induction of G₁ arrest is dependent upon sequence-specific DNA binding and transcriptional activation of p53 target genes such as *p21*^{WAF-1/CIP-1} (hereafter termed *p21*; El-Diery *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; for a review see Gottlieb and Oren, 1996). *p21* is an inhibitor of cyclin dependent kinase (CDK) and blocks the activity of G₁ cyclin-CDK complexes. In cells induction of *p21* can block phosphorylation and inactivation of the retinoblastoma protein, thus contributing to G₁ arrest (Slebos *et al.*, 1994). Other genes up-regulated by p53 may also contribute to cell growth arrest.

Induction of apoptosis is less well understood. Recent work indicates that induction of apoptosis by p53 can be

achieved by both transactivation-dependent and transactivation-independent pathways. Importantly, some transactivation-defective mutants of p53 retain the ability to induce apoptosis (Haupt *et al.*, 1995, 1996; reviewed in Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997).

Missense point mutations can inactivate p53 and this is the most common genetic event linked with human cancer (Cho *et al.*, 1994; Prives, 1994; Hollstein *et al.*, 1996). It is also possible that abnormal regulation of p53 may contribute to the development of those tumours expressing wild-type protein (see Milner, 1995a). However, the molecular mechanisms operating to regulate p53 are poorly understood. It is likely that post-translational modification plays a regulatory role and, indeed, p53 is subject to numerous phosphorylations/dephosphorylations, but the functional significance of such modifications is, as yet, unclear (Meek, 1994; Shaw *et al.*, 1996). One tantalizing observation is that phosphorylation at Ser315, a substrate for CDK phosphorylation, can bias targeting of p53 to specific DNA binding sequences (Wang and Prives, 1995).

Functional regulation may also operate at the levels of tertiary and quaternary structure. The importance of tertiary structure in p53 function is underscored by the precise conformational folding of the core domain that is required in order to position those residues involved in sequence-specific DNA contacts and transactivation of p53 target genes (Cho *et al.*, 1994; reviewed in Friend, 1994; Ko and Prives, 1996). Wild-type p53 has intrinsic flexibility and co-operative conformational shifting between monomers of oligomeric p53 has been demonstrated *in vitro* (Milner *et al.*, 1991) and there is evidence that p53 conformation is subject to cell growth conditions *in vivo* (Milner and Watson, 1990). Additional modifications, involving N- and C-terminal epitopes of p53, are observed during mitogenic stimulation of primary T lymphocytes (Milner, 1984). These various changes may involve post-translational modification(s), interaction with co-factors, conformational shifts and/or protein-protein interactions. Overall they indicate possible allosteric regulation of p53 (Cook and Milner, 1990; Milner *et al.*, 1991; Hupp and Lane, 1994; reviewed in Milner, 1995b).

A crucial step in regulation of p53 concerns its activation in response to DNA damage. This process appears to be independent of gene transcription and may operate at the levels of translation and/or post-translation of p53 protein (Nelson and Kastan, 1994; Fu *et al.*, 1996). Activation is marked by increased levels of cellular p53 and/or increased sequence-specific DNA binding and transactivation of p53 target genes (Tishler *et al.*, 1993; Nelson and Kastan, 1994; Siegel *et al.*, 1995). DNA double-strand breaks can activate p53 and the system appears to be exquisitely sensitive since as little as a single double-strand break may be sufficient to activate a cellular p53 response (Di

Leonardo *et al.*, 1994; Nelson and Kastan, 1994; Huang *et al.*, 1996; reviewed in Kastan, 1996). The p53 protein can bind at sites of DNA damage such as dsDNA ends, ssDNA and insertion/deletion (IDL) mismatches (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994; Jayaraman and Prives, 1995; Lee *et al.*, 1995; Reed *et al.*, 1995). The structural domain of p53 involved in binding to sites of DNA damage is the C-terminal 14 kDa domain. Other stimuli may also serve to activate p53, since it responds to: (i) ribonucleotide depletion in the absence of detectable DNA damage (Linke *et al.*, 1996); (ii) inhibition of RNA polymerase activity (Ljungman and Zhang, 1996). It is also theoretically possible that different agents may activate p53 by different mechanisms.

In order to investigate the activation of p53 we have previously analysed its interaction with dsDNA containing IDL mismatches (lesion or L-DNA) *in vitro*. The p53 protein was purified from *Sf9* insect cells infected with baculoviral expression vectors for either murine or human p53. Initial studies revealed that L-DNA induces proteolytic cleavage of p53 to yield p40 (core + C-terminus) and p35 (core) products (Molinari *et al.*, 1996). The p35 core was conformationally intact and was released from p53–DNA complexes under all conditions tested. We now show that the p35 product has protease activity and is able to cleave the N-terminus of full-length p53 to generate a novel product, p50(Δ N23). Unexpectedly, interaction with ssDNA was found to induce C-terminal cleavage of p53 and generate p50(Δ C) and p40(Δ C). The functional significance of these various p53 cleavage products in relation to the cellular response to DNA damage is discussed.

Results

The observation that interaction with damaged DNA induces proteolytic cleavage of p53 *in vitro* (Molinari *et al.*, 1996) led us to consider that the protein may have intrinsic protease activity. p53 assembles into homologous tetramers and may therefore be susceptible to either intramolecular processing (within a p53 monomeric subunit) and/or intermolecular processing (between neighbouring p53 molecules). In order to investigate the possibility of intermolecular processing we have now asked if p53 has the capacity to cleave other p53 molecules.

p35 cleaves the N-terminus of full-length p53

Purified full-length p53 protein is stable in solution unless incubated in the presence of DNA. Interaction with either L-dsDNA or with ssDNA induces selective and limited cleavage of p53 protein (Molinari *et al.*, 1996; see also Figure 1B). We therefore reasoned that interaction with DNA might somehow activate p53 as a protease and that such activation might involve removal of a negative regulatory domain(s) from the protein. Obvious candidates for screening were the cleavage products generated by interaction with DNA. In this study the p35 core fragment has been tested since it is relatively easy to harvest following its release from p53–DNA complexes.

Biotinylated DNA bound to streptavidin-coated beads was employed in these experiments, since this permits separation of p53–DNA complexes from any products released into the supernatant (Materials and methods).

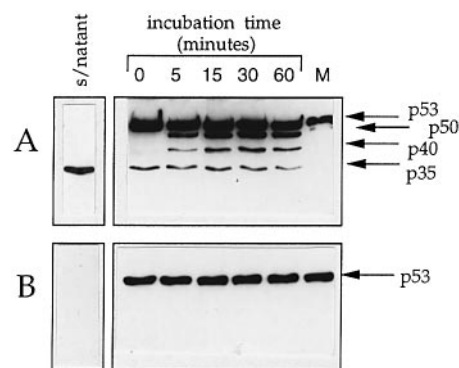


Fig. 1. The p35 core fragment induces cleavage of full-length p53. (A) Freshly prepared p53 protein was mixed with L-dsDNA and p53–DNA complexes were harvested on streptavidin-coated beads, washed and incubated for 60 min. p53–DNA complexes were then pelleted and the supernatant containing the released p35 core fragment of p53 was harvested. An aliquot of the supernatant was reserved for immunoblotting (supernatant lane) and the rest was added to p53 protein in solution and incubated for various times, as indicated. The dilution factor upon addition of supernatant into fresh solution containing full-length p53 was 1 in 7. Samples were analysed by 15% SDS–PAGE and immunoblotted using PAb240 as probe with detection by chemiluminescence (see Materials and methods). (B) Identical to (A) except that the DNA used in the initial incubation reaction was the p53 target sequence CON. Full-length p53 and its cleavage products are indicated by arrows. M, marker p53.

A dsDNA oligonucleotide containing a triple cytosine insertion was used to induce cleavage of p53 purified from *Sf9* insect cells (as detailed in Molinari *et al.*, 1996). Interaction with this L-dsDNA induces: (i) N-terminal cleavage to yield a p40 product which remains bound to the residual full-length p53–DNA complexes; (ii) N- plus C-terminal cleavage to give a conformationally intact core domain of p53 which is released into the supernatant. Freshly purified p53 was incubated with L-dsDNA at 37°C for 60 min, after which time p53–DNA complexes were pelleted and the supernatant harvested. An aliquot of the supernatant was taken for analysis by immunoblotting and, as expected, contained a single band corresponding to the p35 cleavage product of p53 (Figure 1A, s/natant fraction). An equivalent aliquot of the supernatant (containing p35) was added to fresh full-length p53, mixed and incubated at 37°C. Samples were withdrawn from the incubation mix at various times and analysed by immunoblotting, using PAb240 as probe. At the time of mixing the presence of full-length p53 and the p35 cleavage product were evident in the incubation mix (Figure 1A, time zero). Prolonged exposure of the immunoblot failed to reveal any other protein bands (not shown). Subsequently, two additional bands with apparent molecular weights of 50 (p50) and 40 kDa (p40) were clearly detectable between 5 and 60 min incubation (Figure 1A). During this period the amount of p35 core fragment remained essentially unchanged, indicating that p35 is only generated in the presence of damaged DNA. It should be noted that there was a dilution factor of 1 in 7 on addition of p35 to the solution containing full-length p53 (Materials and methods). This accounts for the lower intensity of signal observed for p35 in lanes 0–60 min relative to the supernatant fraction (Figure 1A, s/natant lane).

The above experiment included two negative controls.

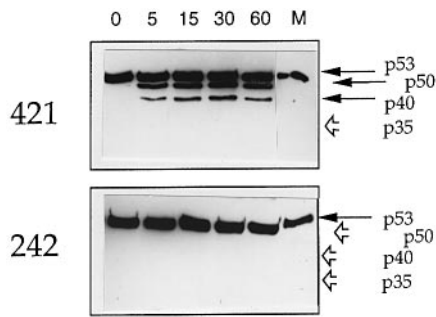


Fig. 2. Determination of the location of p53 cleavage by N- and C-terminal immunoblots (see also Table I). Aliquots of those samples shown in Figure 1 were subject to analyses by further immunoblotting, using PAb242 (against an N-terminal epitope) and PAb421 (C-terminal epitope) as probes. Solid arrows, reactive p53 and its cleavage products; hollow arrows, positions of non-reactive protein bands.

In the first the primary incubation was with CON dsDNA, which contains a specific p53 target sequence and is bound by the p53 core domain (Funk *et al.*, 1992). Sequence-specific binding to CON DNA does not induce proteolytic cleavage of p53 (Molinari *et al.*, 1996) and, as expected, no products were released from the p53–CON DNA complexes (Figure 1B, s/natant fraction). When an aliquot of the supernatant from p53–CON DNA complexes was added to full-length p53 no proteolytic cleavage of the full-length protein was observed over the 60 min incubation period (Figure 1B). Indeed, the full-length protein was stable up to 12 h prolonged incubation (not shown). The second control was designed to check against the unlikely possibility that L-dsDNA bound to streptavidin-coated beads might release some component into the supernatant which is capable of inducing proteolytic cleavage of full-length p53. In these experiments biotinylated L-dsDNA was bound to streptavidin-coated beads and incubated in the absence of p53. An aliquot of the supernatant from this incubation was added to full-length p53, mixed and analysed at various times as described for Figure 1. Again, no proteolytic cleavage of the full-length protein was detectable and the results (not shown) were essentially the same as those presented in Figure 1B.

Overall, these results indicate that the p35 core fragment of p53 has protease activity and is able to cleave full-length p53 and generate p50 and p40 cleavage products. Prolonged incubation did not result in further cleavage of the residual full-length p53 observed in Figure 1A, suggesting that the process is self limiting under the conditions of the experiment.

p50 and p40 are generated by N-terminal cleavage of p53

The results presented in Figure 1 were obtained using the monoclonal antibody PAb240 for immunoblotting. This antibody detects a highly conserved epitope within the central core domain of p53 (residues 212–217). In order to characterize p50 and p40 in more detail we performed additional immunoblots, using PAb242 to detect the N-terminus of murine p53 (residues 18–27). The C-terminus was detected using PAb421 (residues 370–378). The samples taken for immunoblotting were aliquots of those samples previously probed with PAb240 (Figure 1A). The results show that both p50 and p40 are detected by PAb421 (Figure 2, upper panel) but are negative for

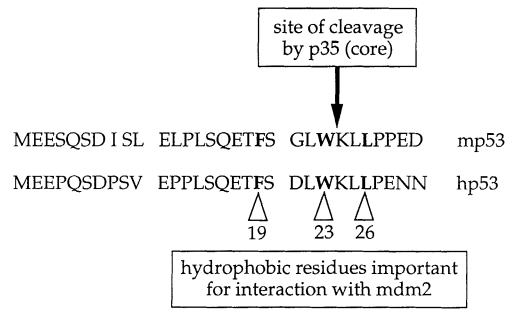


Fig. 3. N-terminal sequences of murine and human p53 showing the site of cleavage of full-length p53 when incubated in the presence of the p35 core fragment (see Figures 1 and 2). This region also contains an α -helical motif with residues important for interfacing with mdm2, a negative regulator of p53 function (Kussie *et al.*, 1996; see Discussion).

Table I. Effects of protease inhibitors on N-terminal cleavage of p53 to generate p53(Δ N23)

Inhibitor	Specificity	Cleavage
Aprotinin	serine proteases	+
Leupeptin	serine and cysteine proteases	+
E64	cysteine proteases	+
Bestatin	aminopeptidase	0
Phosphoramidon	metalloendopeptidases	+

Final concentrations of inhibitors, used according to the manufacturer's instructions (Boehringer Mannheim), were as follows: aprotinin, 2 μ g/ml; leupeptin, 0.5 μ g/ml; E64, 10 μ g/ml; bestatin, 40 μ g/ml; phosphoramidon, 300 μ g/ml.

the PAb242 epitope (Figure 2, lower panel). As expected, full-length p53 was reactive with both N- and C-terminal monoclonal antibodies, whereas the p35 central core fragment was negative for PAb242 and PAb421 (Figure 2; positions of positive bands indicated by solid arrows, positions of non-reactive protein bands indicated by hollow arrows). These results indicate that both p50 and p40 are generated by N-terminal cleavage of p53, hereafter termed p50(Δ N) and p40(Δ N) respectively.

Determination of the N-terminal cleavage site of p50(Δ N) by microsequencing

p50(Δ N) represents a novel cleavage fragment of p53 and the cleavage site was next determined by microsequencing. The results revealed that the site of cleavage was between Trp23 and Lys24; the product is hereafter termed p50(Δ N23). This site and its flanking residues are conserved between murine and human p53 (see Figure 3).

Generation of p50(Δ N23) is blocked by bestatin, an aminopeptidase inhibitor

The generation of p50(Δ N23) proved to be resistant to a range of protease inhibitors (Table I), including aprotinin and leupeptin (inhibitors of serine proteases), E64 and leupeptin (cysteine proteases), pepstatin (aspartate proteases) and phosphoramidon (metalloendopeptidases). However, bestatin, an aminopeptidase inhibitor, effectively blocked generation of p50(Δ N23) (Table I), suggesting that N-terminal cleavage of p53 may involve aminopeptidase activity. Aminopeptidases catalyse cleavage of amino acids from the N-terminus of a protein. Many are zinc metalloenzymes and bestatin represents a transition state

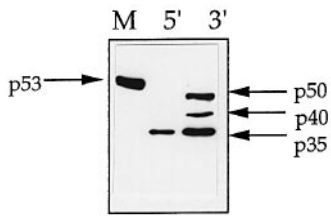


Fig. 4. Cleavage of p53 when incubated in the presence of ssDNA and comparison of the effects of ssDNA with free 3'- and 5'-ends. Identical DNA oligonucleotides were biotinylated at either the 5'-end (3'-free) or the 3'-end (5'-free) and incubated with freshly prepared p53 for 60 min (Materials and methods). After incubation supernatant samples were analysed by immunoblotting using PAb240 as probe. M, full-length p53 marker; 5', ssDNA with 5'-end free; 3', ssDNA with 3'-end free.

analogue which blocks the reaction (Taylor, 1993). In this context it should be noted that the p35 cleavage product of p53 represents a conformationally intact core domain and is predicted to contain a single zinc atom (Cho *et al.*, 1994; Molinari *et al.*, 1996). Since p35 also appears to exhibit protease activity and can cleave the N-terminus of p53 (Figures 1 and 2) we suggest that p35 may be associated with aminopeptidase activity.

C-terminal cleavage of p53 induced by interaction with 3'-free ssDNA

In previous studies we have observed that ssDNA also induces cleavage of p53, with release of p35 from protein-DNA complexes. Others have shown that there are two types of interaction between p53 and ssDNA, with binding at the ends of the ssDNA molecule via the C-terminus of p53 or at internal sites of ssDNA via the core domain (Selivanova *et al.*, 1996). Furthermore, it is possible that the 3'- and 5'-ends of ssDNA may differ in their molecular interaction with p53, since the protein has intrinsic 3'→5' exonuclease activity (Mummenbrauer *et al.*, 1996). The experimental protocol used in our present study employs DNA which has been biotinylated at one end and bound to streptavidin-coated beads. This effectively blocks one end of the ssDNA molecule and we reasoned that this may influence the interaction between p53 and the end of the ssDNA, depending on which end of the ssDNA is available for protein interaction. We therefore compared the effects of 3'- and 5'-biotinylation on proteolytic cleavage of p53. The DNA molecules will be identified by their free ends, 3'-free (5'-biotinylated) and 5'-free (3'-biotinylated), and the same 53mer oligonucleotide was used throughout (see Materials and methods).

Incubation of p53 with 5'-free ssDNA induced release of the p35 cleavage product (Figure 4). No additional cleavage products were detectable and this result is the same as has been reported previously (Molinari *et al.*, 1996). In contrast, a novel cleavage pattern was obtained for p53 incubated with 3'-free ssDNA. With these incubations p50 and p40 products were released from the p53-DNA complexes, in addition to the p35 core domain (Figure 4). Further characterization by immunoblotting with PAb242 and PAb421 revealed that p50 and p40 were generated by C-terminal cleavage of p53 (Table II), hereafter termed p50(ΔC) and p40(ΔC).

Thus, when the 3'-end of ssDNA is available for interaction with p53 we unexpectedly observed C-terminal

Table II. Immunoreactivity of p53 cleavage fragments determined by immunoblotting (see Results)

	242	248	240	421
p53	+	+	+	+
p50(ΔN23)	0	+	+	+
p40(ΔN)	0	0	+	+
p50(ΔC)	+	+	+	0
p40(ΔC)	0	+	+	0
p53 (core)	0	0	+	0
p50(ΔC) ^a	+ ^b		+	0

^aCleavage product induced by treatment of ML-1 cells with adriamycin or cisplatin.

^bDO-1 monoclonal antibody against the N-terminus of human p53.

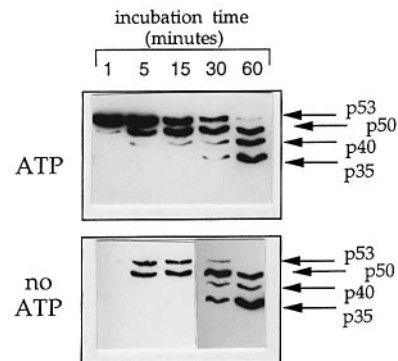


Fig. 5. Time course studies of the release of p53 and its cleavage products from p53-ssDNA complexes. The ssDNA was 3'-end free. Incubations were carried out for various times in the presence or absence of ATP (5 mM) as indicated. Samples were run by 15% SDS-PAGE and probed with Pab240. The results shown in the lower panel (no ATP) are compiled from two gels. The main difference between the upper and lower panels is the release of full-length p53 in the presence of ATP (lane 1 min; see text).

cleavage of the protein. Two novel p50(ΔC) and p40(ΔC) cleavage products were generated and released from the protein-DNA complexes. Overall, these results indicate that p53 interacts differentially with the 3'- and 5'-ends of ssDNA.

Kinetics of p53 cleavage induced by ssDNA in vitro

Time course studies were next carried out in order to determine the sequence of appearance of p53 cleavage fragments following binding to 3'-free ssDNA. The supernatants from p53-ssDNA complexes were harvested at given times and analysed for p53 by immunoblotting with PAb240. Previous studies have indicated that ATP can influence the release of p53 and its cleavage products from DNA (Molinari *et al.*, 1996) and we therefore performed parallel incubations in the presence and absence of ATP. Initial p53-DNA complexes were obtained (Materials and methods) and equivalent aliquots of sample were divided equally and incubated in the presence or absence of ATP (5 mM). Under both conditions of incubation p50(ΔC) was clearly detectable at 5 min and did not appear to decrease or increase upon prolonged incubation up to 60 min (Figure 5). These results suggest that p50 is a stable product of p53 and is generated within less than 5 min of p53 binding to ssDNA.

Longer incubations were necessary before p40(ΔC) and p35 cleavage products were detectable. In the presence of

ATP the p40(Δ C) product was barely detectable at 5 min, but subsequently accumulated to relatively high levels during the 60 min incubation period (Figure 5, upper panel). In the absence of ATP the generation of p40 appeared to be delayed (Figure 5, lower panel), being first detectable at 30 min. The p35 core product of p53 was also detectable at 30 min and here the kinetics were independent of the presence of ATP (Figure 5, compare upper and lower panels; note that these results represent equal exposure times for the immunoblots and are strictly comparable). Interestingly, the appearance of p40(Δ C) and p35 correlated with a decrease in full-length p53 (Figure 5, compare samples taken at 30 and 60 min).

Another point of note in these results is the release of full-length p53 from p53–ssDNA complexes (Figure 5). A high level of p53 in the supernatant was evident within 1 min incubation in the presence of ATP, whereas in the absence of ATP no full-length p53 was detectable until the 5 min incubation period (Figure 5, compare upper and lower panels). Others have shown that p53 binds ATP (Brain and Jenkins, 1994) and it is well established that many DNA binding proteins utilize ATP to promote conformational changes which allow assembly with, or dissociation from a DNA target (Thiagaligam and Grossman, 1993; Cox, 1994; Hegde *et al.*, 1996). No hydrolysis of ATP was detectable in our experiments and ATP did not block p53–ssDNA binding (A.Okorokov and J.Milner, unpublished observations). Our present results thus suggest that ATP may in some way modify the affinity of p53–ssDNA complexes, possibly due to some conformational shift of the protein. It is likely that the increased levels of p53 released into the supernatant may account for the elevated levels of cleavage products obtained in the presence of ATP compared with the absence of ATP (Figure 5, upper panel versus lower panel).

C-terminal cleavage of p53 occurs *in vivo* and correlates with up-regulation of target gene expression

Deletion of 30 residues from the extreme C-terminus of p53 activates its functions as a transcription factor (see Discussion) and C-terminal cleavage may thus provide one mechanism for activating p53 *in vivo* following exposure of cells to DNA damage. In order to test this possibility we treated human ML-1 cells with either adriamycin or cisplatin (Materials and methods) and analysed cellular p53 protein by immunoblotting with different monoclonal antibodies. Following drug treatment a second lower p53 band was detected with PAb240 (Figure 6A upper panel, arrows indicate the p53 doublet) and also with DO-1, which detects an N-terminal epitope on human p53 (results not shown). The mobility of the lower band corresponded approximately with p50(Δ C) and its reactivity with both PAb240 and DO-1 is strong evidence that it is a p53-derived product. Only full-length p53 was detectable with PAb421 (Figure 6A), indicating that the lower protein band lacked the C-terminal 421 epitope of p53. For cells treated with adriamycin the C-terminal cleavage of p53 was first detectable 3 h after treatment and correlated with up-regulation of *p21*, a known target of activated p53 (Figure 6A). Similar results were obtained for cells treated with cisplatin, except that the kinetics were slightly delayed, with C-terminal

cleavage of p53 and induction of *p21* expression occurring between 3 and 6 h exposure to the drug (Figure 6B). Under the conditions of the experiments both adriamycin and cisplatin induced G₁ arrest and apoptosis of ML-1 cells (Figure 6C; F.Ponchel and J.Milner, unpublished observations).

Discussion

p53 is a modular protein and its functional domains have been explored by genetic mutation and deletion analyses. The N-terminus, for example, interacts with cellular transcriptional elements and is necessary for transactivation of p53 target genes. The central core domain (residues 102–292) is also necessary for transcriptional activation and precise conformational folding of this domain is required to orientate those residues which physically interact with specific DNA target sequences (Cho *et al.*, 1994). The C-terminus of p53 contains the oligomerization domain (residues 324–355) which forms dimers and tetramers, formed from a dimer of dimers (Clore *et al.*, 1994). The C-terminus is also involved in recognition and binding at sites of DNA damage and this property is likely to prove crucial for activation of the p53 response (Lee *et al.*, 1995). Once activated, p53 is able to orchestrate numerous cellular control pathways and induce growth arrest in G₁ of the cell cycle. Alternatively, p53 initiates apoptosis. A number of factors influence the choice between growth arrest and apoptosis and recent work indicates that these are genetically separable pathways (Chen, X. *et al.*, 1996; reviewed in Ko and Prives, 1996; Gottlieb and Oren, 1996; Polyak *et al.*, 1996; Rowan *et al.*, 1996; Levine, 1997).

But how is p53 activated in the first place? On the basis of our results we now propose that selective functions of p53 may be activated by proteolytic cleavage of the protein and that this process is triggered by interaction of p53 with sites of DNA damage. Moreover, cleavage can occur either at the N-terminus or at the C-terminus and can be determined by the nature of the DNA which induces the primary cleavage reaction (i.e. L-dsDNA or ssDNA; Molinari *et al.*, 1996; this paper). We have previously discussed the possibility that damaged DNA may act as a co-factor to trigger autoproteolysis of p53 (Molinari *et al.*, 1996) and our present results are consistent with the notion that interaction with damaged DNA may initiate a cascade of proteolytic cleavage steps linked with activation of p53 protein.

N-terminal cleavage and p53 function

We have now shown that the p35 (core) fragment of p53 has protease activity and cleaves the extreme N-terminus of full-length p53 (Figures 1 and 2). Microsequencing revealed that the cleavage site was between Trp23 and Lys24 (Figure 3). Although the N-terminus is required for transactivation of p53 target genes there is evidence that the extreme N-terminus is dispensable for this property. This was clearly demonstrated by Chen, X. *et al.* (1996) who reported that an engineered deletion mutant of p53 lacking the first 22 amino acids (Δ N22) is both transcriptionally active and as potent at inducing apoptosis as wild-type p53. It is likely that p50(Δ N23) may be equally functional and experiments are underway to determine its

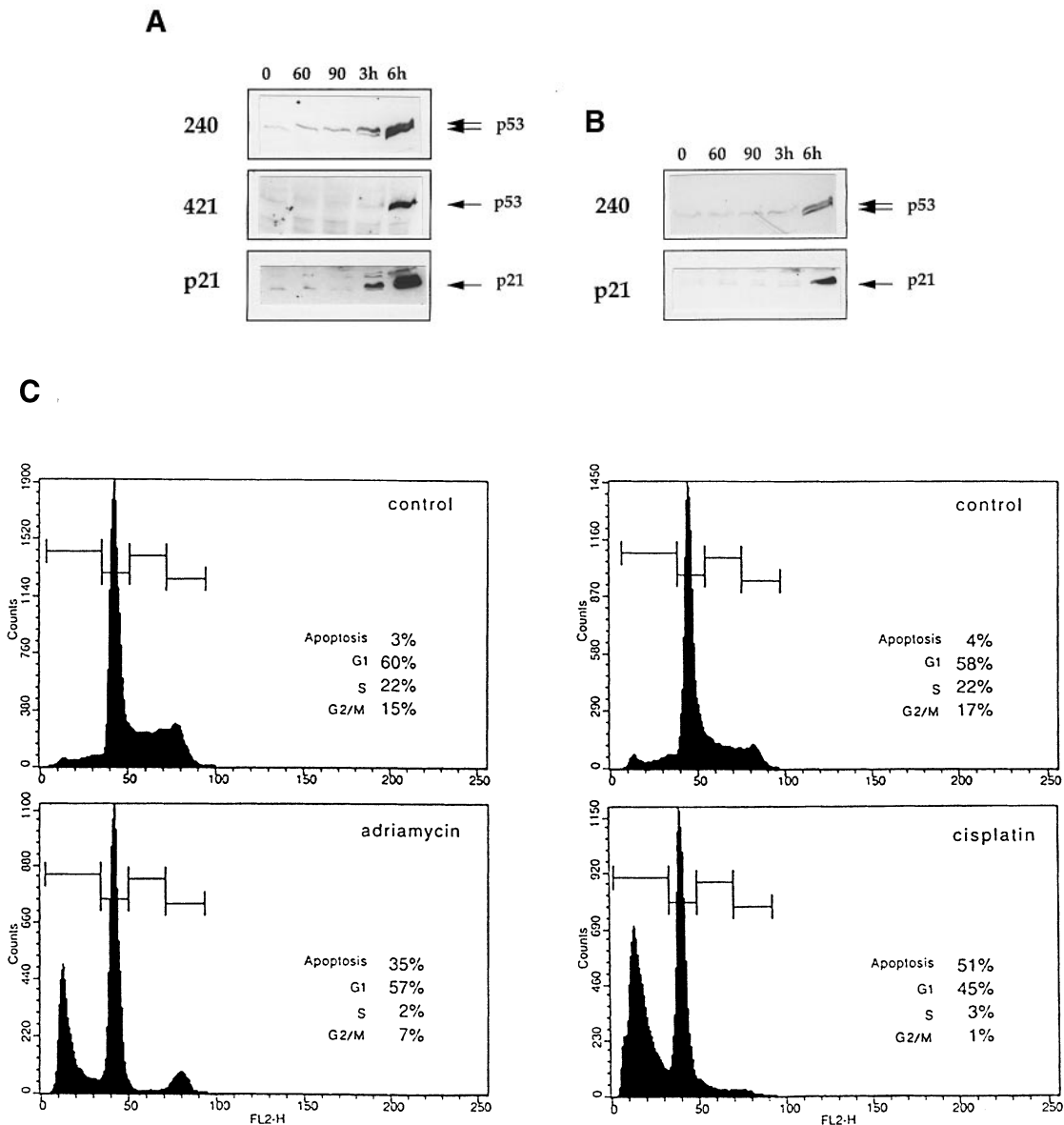


Fig. 6. C-terminal cleavage of endogenous wild-type p53 and up-regulation of p21 expression in ML-1 cells. Cells were treated with either adriamycin (1 μ g/ml) or cisplatin (10 μ g/ml) and analysed for p53 and p21 expression by 15% SDS-PAGE and immunoblotting (Materials and methods; p21 monoclonal antibody was from Santa Cruz, clone sc-817). The double arrows indicate full-length p53 (upper arrow) and the faster migrating p53-derived product (lower arrow) detected by PAb240; the same doublet was observed with DO-1 (not shown), which detects an N-terminal epitope on human p53. Only full-length p53 was detected by PAb421. p53 and p21 proteins are as indicated. Positions of molecular weight and p53 markers are not included in the figure. Cells were harvested at times 0, 60, 90, 180 and 360 min after treatment with adriamycin (A) or with cisplatin (B) as indicated. (C) Cell cycle analysis of ML-1 cells before and after 24 h treatment with adriamycin and cisplatin as detailed in Materials and methods. Bars indicate gating for apoptotic, G₁, S and G₂/M cells respectively and are based on DNA content. Apoptosis was confirmed by labelling of cells with annexin V.

transactivation potential and ability to induce apoptosis in transfected cells.

However, p50(Δ N23) is almost certain to differ from the intact protein in at least one important respect. This concerns the protein-protein binding between p53 and mdm2, a negative regulator of p53 transactivation and apoptotic activity (Lin *et al.*, 1994; Chen, J. *et al.*, 1995, 1996; Haupt *et al.*, 1996). The crystal structure of the p53-mdm2 interface shows that interaction relies on steric complementarity between mdm2 and p53. In particular, this involves residues Phe19, Trp23 and Leu26, which are located on the hydrophobic face of the p53 α -helix (Kussie *et al.*, 1996). Removal of both Phe19 and Trp23 is

therefore predicted to seriously impair the ability of mdm2 to bind p50(Δ N23). It follows that transactivation of target gene expression by p50(Δ N23) is likely to be resistant to down-regulation by mdm2. This is represented schematically in Figure 7.

A second N-terminal cleavage site generates p40(Δ N) and involves loss of the transactivation domain (within residues 1-42; Unger *et al.*, 1993) and removal of the epitope recognized by PAb248 (residues 44-60; see Molinari *et al.*, 1996; Table I). This cleavage is predicted to enhance transcriptional repression and also the DNA and RNA reannealing properties of p53 (Brain and Jenkins, 1994; Horikoshi *et al.*, 1995). It should be noted that p53

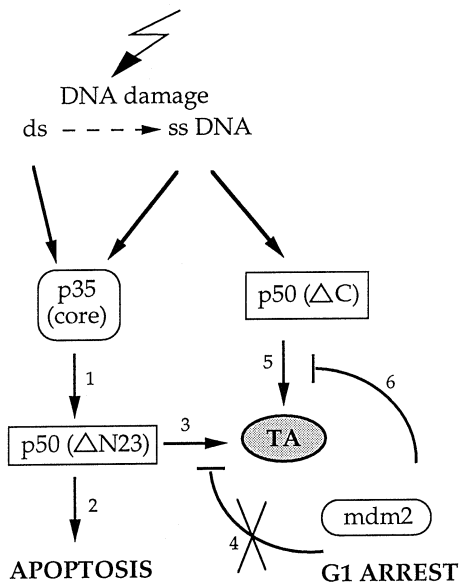


Fig. 7. Schematic model for activation of p53 in response to DNA damage and for the possible roles of the p35 and p50 cleavage products released from p53–DNA complexes. Both L-dsDNA and ssDNA induce cleavage of full-length p53 to produce the p35 core fragment (Molinari *et al.*, 1996; this paper). In its turn p35 promotes N-terminal cleavage of full-length p53 to generate p50(ΔN23) (arrow 1). This N-terminal truncated product may have the same capacity to transactivate p53 target genes and induce apoptosis as ΔN22 (arrows 2 and 3; Chen,X. *et al.*, 1996; see Discussion). However, p50(ΔN23) has lost part of the interface for mdm2 binding and may therefore be resistant to mdm2 inhibition (step 4). Incubation with ssDNA also induces C-terminal cleavage of p53, known to activate its ability to transactivate target genes and induce G₁ arrest (arrow 5; Hupp *et al.*, 1992; Chen,X. *et al.*, 1996). One target gene is *mdm2*, which down-regulates p53 by binding to the N-terminus (step 6). If the concentrations of p53 cleavage products reflect the magnitude of DNA damage these alternative pathways might influence the determination between cell growth arrest or apoptosis. TA, transcriptional activation.

can be a substrate for proteolytic cleavage by calpains (Kubbutat and Vousden, 1997; Pariat *et al.*, 1997) with an N-terminal cleavage site. However, calpain cleavage of p53 is inhibited by chelating agents (Pariat *et al.*, 1997) and is therefore unrelated to the cleavage induced by damaged DNA, since the latter is resistant to the presence of chelating agents (Molinari *et al.*, 1996).

C-terminal cleavage

The last 30 residues of p53 constitute a negative regulatory domain with potent suppression of sequence-specific DNA binding by p53 and transactivation of target genes. Deletion of the C-terminus activates specific DNA binding *in vitro* and transactivation of target gene expression in transfected cells (Hupp *et al.*, 1992; Halazonetis and Kandil, 1993; Cox *et al.*, 1995; Pellegata *et al.*, 1995; Chen,X. *et al.*, 1996). Using an inducible expression system Chen,X. *et al.* (1996) showed that p53(ΔC30) up-regulates expression of *p21* as efficiently as the wild-type protein. Interestingly, however, ΔC30 was much less effective than wild-type in induction of apoptosis. Similar results were recently obtained for alternatively spliced murine p53 (p53AS) (Almog *et al.*, 1997). These observations indicate that the C-terminal amino acids of p53 are necessary for efficient apoptotic activity.

In this paper we report that interaction with ssDNA

induces C-terminal cleavage of p53 to generate p50(ΔC). Based upon the change in molecular weight we calculate a loss of ~30 residues from the C-terminus. A similarly sized product of endogenous wild-type p53 was observed *in vivo* following exposure of cells to adriamycin or cisplatin (Figure 6). This p53-derived product lacked the C-terminus and its appearance correlated with cellular up-regulation of *p21*. We therefore suggest that p50(ΔC) is a natural cleavage product of p53 and may be activated for transactivation of p53 target genes *in vivo*. The p50(ΔC) cleavage product has an intact N-terminus and should therefore retain sensitivity to mdm2 feedback inhibition (see Figure 7).

Different types of DNA 'damage' induce different p53 cleavage products

Interaction with L-dsDNA induces N-terminal cleavage of p53 (Molinari *et al.*, 1996) and it was therefore a surprise when we discovered that ssDNA induces C-terminal cleavage under identical conditions (Results). This led us to review in detail the literature relating to the functional properties of truncated p53 proteins generated by deletion mutagenesis of p53. From these studies we have developed a provisional model for the putative role(s) of proteolytic cleavage in the p53 response to DNA damage (summarized in Figure 7).

We propose that the ability of p53 to exhibit a differential response to different types of DNA damage may be fundamentally important in cell growth control and may influence the choice between cell growth arrest and apoptosis. For example, repair of damaged DNA involves mechanisms such as nucleotide excision repair and involves ssDNA intermediates (Huang *et al.*, 1992; Huang and Sancar, 1994). By inducing C-terminal cleavage of p53 (and transactivation of p53 target genes) these single-stranded intermediates would contribute towards the maintenance of G₁ arrest during the repair process. Thus the period of cell growth arrest would be tailored to fit the time required for DNA repair. Moreover, since the C-terminus of p53 appears important for an efficient apoptotic response (Chen,X. *et al.*, 1996), its removal will minimize the apoptotic potential of p50(ΔC) and favour cell growth arrest.

If, on the other hand, a cell sustains heavy damage to its DNA, the balance may be tipped towards apoptosis by N-terminal cleavage of p53 to produce p50(ΔN23). As discussed above, p50(ΔN23) is likely to be competent for transactivation of p53 target genes and for induction of apoptosis. However, it has lost a major part of the mdm2 binding interface (hydrophobic residues Phe19 and Trp23) and may therefore be resistant to feedback regulation by mdm2. Under these conditions p50(ΔN23) may promote apoptosis (see Figure 7). The apoptotic pathways may be preceded by a transient growth arrest, reflecting up-regulation of target genes such as *p21*. Indeed, this is exactly what is observed when ΔN22 is expressed in cells (Chen,X. *et al.*, 1996). A key player in this scenario is p35, the core fragment of p53 which is released from p53–DNA complexes. In this paper we show that p35 has protease activity and is able to cleave the N-terminus of p53 to generate p50(ΔN23). In this way p35 may serve to reinforce the p53 response to cellular DNA damage.

In summary, intact p53 protein appears to encompass a

number of cryptic functions which can be selectively activated by specific truncations or deletions of the polypeptide. In some way these same functions are activated in response to DNA damage. We have discovered that interaction with damaged DNA induces selective proteolytic cleavage of the p53 protein, with removal of known negative regulatory domains from the protein. Based upon our observations we present a model for activation of p53 by proteolytic cleavage in response to DNA damage. This model would provide a simple yet elegant means for activation of p53 following DNA damage and also for regulating the choice between cell growth arrest and apoptosis.

Materials and methods

Oligonucleotides

The following biotinylated oligonucleotides were employed: p53 consensus (CON, 20 bp), 5'-GGACATGCCCGGGCATGTCC-3' (Funk *et al.*, 1992); L-DNA, 5'-GGCTCGAACCCGTTCTCGGAGCACCCCTGCCAGCCCAACCGCTTTGGCCCGCCAGCC-3' (62b), triple cytosine lesions are underlined (Lee *et al.*, 1995). The oligonucleotides were annealed as follows: pCON to itself, L-DNA oligonucleotide to the biotinylated reverse sequence 5'-GGCTGGGCGGGCCAAAGC-GGTTCTGCAGTGCTCCGAGAACCAGGGTTCCGAGCC-3' (53b). The latter was used as an ssDNA template for the experiments when p53 was exposed to ssDNA. According to the experiment it was biotinylated on the 5'- or 3'-end.

Baculoviral expression and purification of p53

Human and murine p53-producing recombinant baculoviruses were constructed and amplified as described in Molinari *et al.* (1996). Shortly-*Sf9* insect cells at 80% confluency in TNM-FH medium (Pharmingene) were infected with high titre recombinant baculovirus and harvested 3 days later. After washing four times with phosphate-buffered saline (PBS) the cell pellet was resuspended and lysed for 30 min on ice in 5 ml lysis buffer (150 mM Tris-HCl, pH 9.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 10 µg/ml pepstatin A and 1 mM β-mercaptoethanol). The lysate was centrifuged at 20 000 r.p.m. in a Sorvall SS-34 rotor at 4°C for 30 min and loaded on a 0.5 ml Ni-NTA agarose column (Qiagen Inc.) prewashed with lysis buffer, pH 7.0. The column was washed with 10 ml lysis buffer, pH 9.0, and adjusted to pH 7.0 with 10 ml lysis buffer, pH 7.0. The column was step-eluted by one volume portions of elution buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 5 mM MgCl₂) containing increasing concentrations of imidazole (50, 100, 150 and 250 mM). The second and third elutions with 250 mM imidazole were used for analyses. Imidazole was removed using Centricon-10 cartridges (Amicon) by washing with imidazole-free elution buffer. p53 protein was quantitated by Bradford assay and purity checked by SDS-PAGE and silver staining. A single band corresponding to p53 was observed.

DNA-dependent proteolysis assay

Streptavidin-coated magnetic beads were used to harvest biotinylated DNA-protein complexes. dsDNA or ssDNA oligonucleotides were bound to the beads (typically 75 pM oligonucleotide/40 µl beads/reaction) in TE, pH 7.5, 1 M NaCl (TE/NaCl) for 15 min at 20°C, washed twice in 400 µl TE/NaCl and twice in 400 µl DNA binding buffer [20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% NP40, 10% glycerol and 5 mM dithiothreitol (DTT)]. The supernatant was replaced by 50 µl fresh DNA binding buffer and 10 µl (10 pM) purified p53 (in 50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 5 mM MgCl₂ and 5 mM DTT) was added to each reaction. Typically 40 µl beads were used in a total reaction volume of 50 µl. After 20 min incubation at 20°C the p53-DNA complexes were collected, washed three times with 400 µl and resuspended in 50 µl DNA binding buffer and incubated at 37°C for 1 h. Reaction products were analysed by 15% SDS-PAGE followed by immunoblotting or were used for the following experiments. For microsequencing the above reactions were scaled up, proteins transferred onto Problot membrane and stained with Coomassie for no longer than 1 min. Relevant bands were excised for microsequencing by standard methods, using a Perkin Elmer microsequencer.

Immunoblotting

Reaction samples were separated by SDS-PAGE and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, 128 mM glycine, 20% methanol and 0.1% SDS) overnight at 18 V. The membrane was washed in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and incubated for 1 h in 1% blocking solution (Boehringer Mannheim) in TBS. The membrane was incubated for 1 h with anti-p53 monoclonal antibodies (Results), washed twice with 100 ml TBST for 15 min and twice with 30 ml 0.5% blocking solution in TBS 0.1% Tween 20. Incubation with the secondary antibody [HRP-conjugated rabbit anti-mouse (Dako), 1:3000 in 0.5% blocking solution/TBS] was for 1 h, followed by four 15 min washes in TBST. Detection was performed using the Boehringer Mannheim chemiluminescence blotting kit.

Cell culture and cell cycle analysis

ML-1 cells (derived from a human myeloblastic leukaemia and expressing wild-type p53) were cultured at 37°C in 5% CO₂ in air in RPMI 1640 supplemented with 10% fetal calf serum, 100 µg/ml penicillin and 100 µg/ml streptomycin. Aliquots of 10⁶ cells were treated with adriamycin (1 µg/ml) or with clinical grade cisplatin (10 µg/ml) as indicated in the text. For immunoblotting cells were harvested, washed in PBS and lysed for 15 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Triton and 5 mM DTT) containing phenylmethylsulfonyl fluoride (1 mM), leupeptin (2 µg/ml) and aprotinin (2 µg/ml) as protease inhibitors. Protein concentrations were determined by the Bradford reaction and equivalent aliquots were subject to SDS-PAGE and immunoblotting as detailed in the text. For cell cycle analysis the cells were washed in PBS and fixed in ice-cold methanol (70% in PBS) for 20 min at -20°C. The cells were then resuspended in 1 µg/ml RNase in PBS and incubated at 37°C for 30 min. Propidium iodide (50 µg/ml) was then added and cell cycles were analysed using a FACSsort (Becton-Dickinson). Apoptotic cells were assessed by a combination of DNA content and by labelling with annexin V.

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