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On the regulation of the p53 tumour suppressor, and its role in the cellular response to DNA damage

DAVID P. LANE[1], CAROL A. MIDGLEY[1], TED R. HUPP[1], XIN LU[2], BORIVOJ VOJTESEK[3] AND STEVEN M. PICKSLEY[1]

[1] *CRC Cell Transformation Group, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K.*
[2] *Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG, U.K.*
[3] *Masaryk Memorial Cancer Institute, Zluty Kopec, 656 53 Brno, Czech Republic*

SUMMARY

The p53 gene is required for the normal apoptotic response of mammalian cells to DNA damage caused by ionizing radiation and DNA damaging drugs. DNA damage results in the accumulation of biologically active p53. This response is potentially lethal and is therefore highly regulated. By using both biochemical and cell biological approaches a number of discrete control pathways have been identified. These include analysis of cellular and viral proteins that bind to p53 to inactivate its function, the discovery of cells with defects in the p53 activation pathway and the analysis of an allosteric regulation of p53 function controlled by phosphorylation.

1. INTRODUCTION

The genetic material of all living organisms is continuously being damaged both by natural chemical processes and by exogenous agents such as chemicals and ionizing radiations present in the environment. Organisms are protected from the mutagenic affects of this barrage by efficient DNA-repair processes. Inherited defects in these repair processes result in a predisposition to the development of cancer. Recently it has become clear that, in addition to the genes involved in the mechanics of repair itself, other genes may also play a crucial role in protecting the organism from the effects of DNA damage. These genes seem to act by preventing the division of cells that have sustained DNA damage. One of this latter group of genes is that encoding the p53 protein. Experiments with mice that lack functional p53 genes has shown that they are extraordinarily predisposed to the development of cancer and that their cells are resistant to radiation-induced programmed cell death (Donehower *et al.* 1992; Clarke *et al.* 1993; Lowe *et al.* 1993). The p53 protein acts to induce cell death after its activation in response to DNA damage.

Loss of p53 function is a key step in the development of most human cancers. In most cases these tumours have lost one p53 allele completely but continue to express the other parental allele. Closer analysis shows that this remaining expressed allele contains an inactivating point mutation. The response that p53 induces after DNA damage must be highly regulated to ensure that it is selective and efficient. Three mechanisms of regulation have so far been discovered. Firstly, both cellular and viral gene products have been discovered that will bind to p53 and inactivate its function in the induction of a DNA damage-induced growth arrest. Of particular interest is the *mdm2* gene, because overexpression of the MDM2 protein is frequently found in some human sarcomas. Secondly, p53 is regulated by the control of protein stability. The protein normally has a very short half-life and is present in minute quantities. However, it accumulates rapidly in cells subjected to DNA damage (Maltzman & Czyzyk 1984; Lu & Lane 1993). This response appears to be highly regulated so that on whole-body irradiation, only certain populations of cells accumulate detectable levels of p53. In the small intestine this population of cells exactly coincides with the stem cells in that zone where most apoptosis is seen (Merritt *et al.* 1994). Finally, biochemical evidence suggests that p53 is subject to a very exact allosteric regulation, controlled by phosphorylation of multiple sites within a novel C-terminal regulatory domain. All these control processes are involved in a critical cellular decision process, principally whether to permit survival, or programme cell death, after DNA damage. Understanding this decision process and its regulation in normal and tumour cells will allow a deeper understanding of cancer therapies based on the use of ionising radiation and DNA damaging drugs. It may also shed some light on the mechanisms by which the cell monitors the success of its DNA repair processes.

2. MATERIALS AND METHODS

(*a*) *Pepscan analysis of the MDM2 binding site on p53*

Human and murine p53 was synthesized as a set of overlapping peptides composed of 15 amino acids. Each

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peptide overlapped the previous one in the series by five amino acids and each peptide was synthesized with an N-terminal tail of four spacer amino acids, the last of which was biotinylated. The panel of peptides was arrayed on 96-well microtitre plates coated with streptavidin and then incubated with extracts containing MDM2 protein. The bound MDM2 was then detected by an enzyme-linked immunosorbent assay (ELISA) method by using a mouse monoclonal anti-MDM2 antibody and a peroxidase conjugated anti-immunoglobulin as previously described (Picksley *et al.* 1994).

(*b*) *Defects in the accumulation of p53 after DNA damage*

Primary fibroblasts where obtained from the Human Genetic Mutant Cell Repository (Cornell Cell Reposiformes, New Jersey, U.S.A.) Their ability to accumulate p53 protein after exposure to DNA damaging agents and SV40 virus infection was measured by quantitative p53 ELISA and by indirect immunohistochemistry using a panel of anti-p53 monoclonal antibodies as previously described (Lu & Lane 1993).

(*c*) *Allosteric regulation of p53 function*

The p53 protein was purified by chromatography on heparin agarose, ion exchange and molecular sieve columns as recently described from both baculovirus and *E. coli* expression systems. Its activity as a sequence-specific DNA binding protein was measured in a gel retardation assay (Hupp *et al.* 1992, 1993).

3. RESULTS AND DISCUSSION

(*a*) *Identification of the MDM2 binding site on p53 by using synthetic peptides*

The MDM2 protein can form a tight protein–protein complex with p53 (Barak & Oren 1992; Momand *et al.* 1992). When the MDM2 protein binds p53 it acts to neutralize the activity of p53 as a sequence-specific transcription factor (Momand *et al.* 1992; Oliner *et al.* 1993; Wu *et al.* 1993; Zauberman *et al.* 1993). This is thought to be the mechanism by which MDM2 can act as a dominant transforming oncogene. This is because, in tumours where the gene encoding MDM2 is amplified, p53's tumour suppressor function is neutralized (Oliner *et al.* 1992). Consistent with this, high levels of MDM2 can block the p53-dependant G1 growth arrest induced by ionizing radiation. It is possible that MDM2 may have a natural physiological role in down-modulating the p53 response as it was recently found that the MDM2 gene is positively regulated by p53 (Barak *et al.* 1993; Wu *et al.* 1993). This then sets up a feedback loop between p53 and MDM2 (figure 1), and may also represent a paradigm for the regulation of growth control by p53 (reviewed in Picksley & Lane 1993).

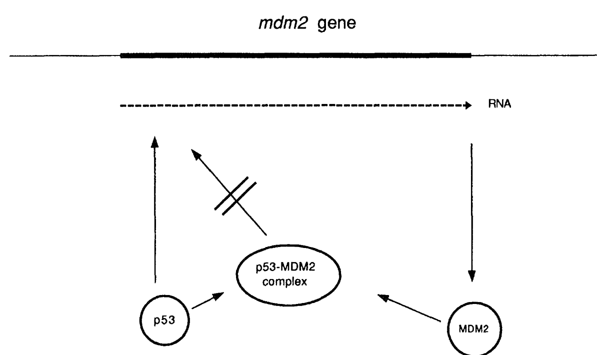

Figure 1. The p53-MDM2 feedback loop (adapted from Wu *et al.* 1993).

A small molecule that could disrupt the p53–MDM2 interaction would have therapeutic potential. It might be used to liberate active wild-type p53 from those tumours that have amplified the MDM2 gene and thus contain no free active p53. It might also be used to affect the duration of the p53 response, for example in response to radiation therapy, perhaps increasing the degree of apoptosis induced at a given dose of radiation. To identify such a small molecule it is important to localize and characterize as precisely as possible the site of interaction between p53 and MDM2. Early work using yeast two hybrid screens and fragments of p53 produced by using recombinant DNA methods had localized the MDM2 binding site to the N-terminal 42 amino acids of p53 (Oliner *et al.* 1993). This corresponds to the region of p53 known to be able to act as a transcriptional transactivation domain (Unger *et al.* 1992). Although these recombinant DNA-based approaches are very powerful they become labour intensive when high resolution is required. As an alternative we have begun to explore the use of monoclonal antibodies, synthetic peptide libraries and phage peptide display libraries to study protein–protein interactions.

By using a panel of monoclonal antibodies raised to p53 to immunoprecipitate p53 in the free form, or p53 complexed to MDM2, it was found that, although antibodies to the C-terminus of p53 could recognize both the free form and the MDM2 complex, an antibody Bp53-19 directed to the N-terminus of the protein could not immunoprecipitate p53 when it was bound to MDM2 but could immunoprecipitate the free protein (Picksley *et al.* 1994).

To localize precisely the binding site for this antibody on p53 it was tested for its ability to interact with a set of overlapping synthetic peptides that each contained 15 amino acids of the human p53 open reading frame. These peptides contain a four amino acid spacer group, the terminal amino acid being biotinylated. Each peptide in the series overlaps the previous one by ten amino acids so that the series progresses in steps of five amino acids. The peptides were displayed using streptavidin coated microtitre wells. A strong and specific signal was obtained with peptides four, five and six when the antibody was used to probe the peptide array. This mapped the binding site of the antibody to the region of overlap between these three peptides: namely the sequence DLWKL which is amino acids 21–25 of human p53 (figure 2).

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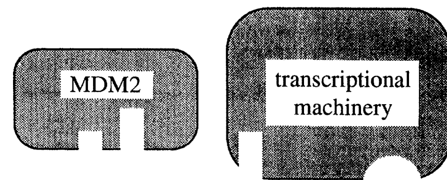
no.	p53 peptide series	MAb Bp53-19 binding	MDM2 binding
1.	MEEPQSDPSVEPPLS	-	-
2.	SDPSVEPPLSQETFS	-	-
3.	EPPLSQETFSDLWKL	+	+
4.	QETFSDLWKLLPENN	+	+
5.	DLWKLLPENNVLSPL	+	-
6.	LPENNVLSPLPSQAM	-	-

▭ antibody epitope overlap
▭ MDM2 binding overlap

Figure 2. Peptides that are recognized by monoclonal antibody Bp53-19 or that are bound by MDM2 protein.

Because the peptide array had worked so well for the mapping of the antibody binding site it seemed reasonable to try and see if MDM2 protein itself would bind to any of the peptides. The MDM2 protein was produced by using a baculovirus-based expression vector and the insect cell extract incubated with the peptide coated wells. To detect any MDM2 protein remaining bound after extensive washing, the wells were then treated with an anti-MDM2 antibody. This antibody was finally detected using a peroxidase conjugated rabbit anti-mouse IgG. Two peptides, numbers three and four in the series, gave a strong signal. On this basis we were able to localize the MDM2 binding site on p53 to the ten amino acids QETFS-DLWKL (figure 2). This represents amino acids 16–25 of human p53 (the same region of mouse p53 also bound MDM2). This site is coincident with, but extends beyond, the binding site for the antibody lending strong support to the conclusion that this is a key area of the molecule for the MDM2 interaction.

To examine the specificity of the interaction in more detail we had peptides synthesized in which each residue of the motif was in turn replaced with alanine. The results obtained with this alanine scan series identified the six amino acids TFSDLW (amino acids 18–23) as being completely intolerant to substitution by alanine if MDM2 binding activity was to remain detectable. The identification of the MDM2 binding site has proved to be of particular interest in the light of recent point-mutational studies carried out by other groups. Dr Vousden's laboratory has found that deletion of residues 13–19 of p53 inactivates p53's ability to bind MDM2 consistent with the above peptide data, but leaves the p53 protein still competent to act as a transcription factor (Marston *et al.* 1993). However, Dr levine's laboratory has found that the double point mutation of residues 22 and 23 (22L to 22Q and 23W to 23S) completely inactivates the transcriptional activity of p53 (Lin *et al.* 1994). These two residues of p53 have been completely conserved in all vertebrate species whose p53 sequence is known. Because our binding data show that these residues are required for MDM2 binding it becomes apparent that the MDM2 binding site physically overlaps the putative binding site for an essential transcription factor that p53 will interact with. It is interesting that this same region of p53 is also recognized by the Adenovirus E1b 55 kDa protein that is also known to block p53 function in transcription assays. A model for these interactions is shown in figure 3.



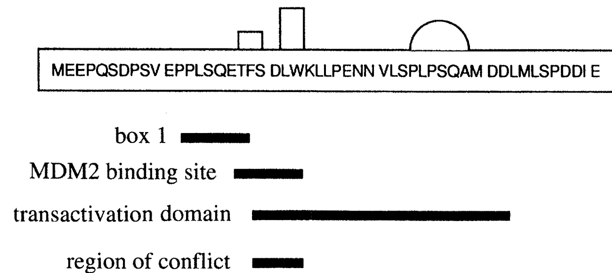


Figure 3. A diagram representing the MDM2 binding domain and the transactivation domain at the N-terminus of p53, and the shared region of interaction/region of conflict.

Using these results it may be possible to devise small molecules that could disrupt specifically the interaction of p53 with MDM2 leaving its ability to interact with transcription factors intact. On a broader front the use of biotinylated peptide sets seems to offer special advantages for probing protein–protein interactions.

(*b*) *Defects in the accumulation of p53 after DNA damage*

The accumulation of p53 protein in cells exposed to DNA damaging agents has been seen using both qualitative immunostaining techniques and quantitative ELISA and immunoblotting assays (Lu & Lane 1993). The principal mechanism responsible for the accumulation seems to be post transcriptional as the response is not inhibited by agents that block transcription (Maltzman & Czyzyk 1984). The accumulated p53 in cells exposed to DNA damage has a longer half life than p53 produced in untreated cells so that regulation of p53 degradation is likely to be a major mechanism involved in the response. It is also possible that the p53 mRNA is preferentially translated in DNA damaged cells as it is known that selective translational control can be an important regulatory mechanism in other stress responses such as heat shock. The induction of p53 accumulation can be seen in whole animals (Hall *et al.* 1993) as well as in tissue culture systems. The response seems to be regulated by cellular factors, as well as by dose, because for example p53 accumulation in the cells of the small intestine is not uniform following exposure to ionizing radiation (Merrit *et al.* 1994). The accumulation of p53 can be directly induced by double strand breaks since introduction of a restriction enzyme into mammalian cells will cause p53 to accumulate (Lu & Lane 1993). The response is blocked by exposure of cells to caffeine which also suggests that it can be modulated by the cellular environment. To begin to identify the pathway and mechanism by which p53 accumulates after DNA damage we screened primary fibroblasts from individuals with a genetic predisposition to genetic

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instability. Although in contrast to the reports of others (Kastan *et al.* 1992) we found no exceptional defect in fibroblasts from patients with ataxia telangiectasia (AT) we did discover that fibroblasts derived from two out of eleven Blooms syndrome patients had a profoundly defective response (Lu & Lane 1993). These cells failed to accumulate p53 normally in response to ionizing radiation, UV radiation or SV40 infection. However, when fused to normal mouse cells they were able to accumulate human p53 after UV radiation and both northern blotting experiments and more recently polymerase chain reaction (PCR)-based methods suggest that they contain normal amounts of p53 mRNA. The defect in these cells is therefore probably not in the p53 gene itself as it can be complemented in trans. If that is the case then it would imply that all three signals for p53 accumulation have a common mechanistic step that is defective in these cells. We are attempting to identify the defect in these cells using cDNA expression libraries. Inactivation of such a gene function would be expected to produce a cancer susceptibility and so we are currently looking for variation in the p53 response among the normal human population and in inbred strains of mice.

(*c*) *Allosteric regulation of p53 function*

The sequence-specific DNA binding function of p53 is important for its tumour suppressor function (Kern *et al.* 1992). p53 binds to DNA elements in gene products which are synthesized after DNA damage linking the biochemical activity of p53 to the DNA damage response. Of particular interest is the discovery that the cell-cycle kinase inhibitor p21/WAF-1 is transcriptionally regulated by p53 (El-Deiry *et al.* 1993). The recently solved crystal structure of the conserved core DNA-binding domain of p53 consists of a novel β-sandwich that functions as a scaffold for two large loops and a loop-sheet-helix motif that constitute the DNA binding interface (Cho *et al.* 1994). A single point mutation within this protease-resistant core DNA binding domain inactivates p53 sequence-specific DNA binding function and results in a global de-stabilization in the proper folding of the core domain (Vojtesek *et al.* 1994).

The biochemical studies aimed at understanding how this sequence-specific DNA binding function of wild-type p53 is regulated have revealed two important control processes. The first involves a form of autoregulation dependent upon a C-terminal regulatory motif which functions to lock p53 tetramers in a biochemically latent state (Hupp *et al.* 1992). Removal of this motif results in the constitutive activation of the protein. This modification can occur in a cell cycle regulated manner as a result of alternate splicing, at least in rodent cell systems (Kulesz-Martin *et al.* 1994). The inhibitory activity of the C-terminal motif can, however, be neutralized *in vitro* by post-translational modification, specifically by phosphorylation. The second point of regulation involves the function of two distinct protein kinases (casein kinase II and protein kinase C) which can activate allosterically the latent sequence-specific DNA binding function of p53 *in vitro* (Hupp & Lane 1994). These latter results suggest that p53 may be regulated by distinct signalling pathways, which is consistent with the central role played by p53 in growth control. What is the physiological significance of the activation of latent p53? Work in our laboratory has demonstrated that the latent form of p53 is synthesized in a variety of eukaryotic cell lines, as its phosphorylation and subsequent activation *in vivo* appears to be rate-limiting. The inability of cells to catalyse complete activation of p53 may relate to the tight regulation of the activity of this protein. Because expression of active p53 is not compatible with cell survival, the cell may have evolved a mechanism to ensure that large levels of active protein are not switched on unless required as part of the response to DNA damage. Experiments with antibodies directed to the negative regulatory motif at the C-terminus of p53 have shown that some mutant p53 proteins retain a latent DNA binding function which could in theory be reactivated to therapeutic effect, restoring the function of wild-type p53 to tumour cells (Hupp *et al.* 1993). These biochemical studies on the regulation of p53 function offer another route by which to discover the pathways that link the growth arrest and apoptotic responses to the DNA damage detection and repair pathways.

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