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DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization

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Upon DNA damage, p53 has been shown to be modified at a number of N-terminal phosphorylation sites including Ser15 and -33. Here we show that phosphorylation is induced as well at a novel site, Ser20. Phosphorylation at Ser15, -20 and -33 can occur within minutes of DNA damage. Interestingly, while the DNA-binding activities of p53 appear to be dispensable, efficient phosphorylation at these three sites requires the tetramerization domain of p53. Substitution of an artificial tetramerization domain for this region also permits phosphorylation at the N-terminus, suggesting that oligomerization is important for DNA damage-induced signalling to p53.
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Introduction

The tumor suppressor protein p53, which is an important regulator of cell cycle and genome stability, is frequently mutated, in >50% of tumors (Hollstein *et al*., 1996). While p53 normally exists in cells as a short-lived protein, in response to stress from DNA damage, hypoxia, nucleotide depletion or activated oncogenes it is stabilized mainly through post-transcriptional mechanisms, which then lead to either growth arrest or apoptosis (reviewed by Levine, 1997; Agarwal *et al*., 1998).

p53 is a sequence-specific transcriptional activator and several functional domains have been identified within the protein. The transactivation domain and a region containing several copies of the motif PXXP which facilitates induction of apoptosis are located within the N-terminus. The central portion contains the core DNA-binding domain which recognizes the sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′. Within the C-terminal region is the tetramerization domain and a highly basic region at the very end of p53 that binds non-specifically to DNA and negatively regulates specific DNA binding by the core domain (reviewed by Ko and Prives, 1996). A number of p53 downstream targets have been identified including the $p21^{WAF1}$, bax, IGFBP3, cyclin G genes and several others (reviewed in Gottlieb and Oren, 1996; Ko and Prives, 1996). In addition, the *mdm2* gene, which encodes a crucial negative regulator of p53 transactivation (Momand *et al*., 1992; Oliner *et al*., 1993) and stability (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Midgley and Lane, 1997) is itself a transcriptional target of p53.

How p53 responds to stress signals is intriguing and not yet fully understood. The possible involvement of p53 modification has long been a subject of intense investigation (reviewed by Giaccia and Kastan, 1998; Prives, 1998; Meek, 1998). p53 has been shown to be post-translationally modified by phosphorylation (reviewed by Meek, 1998), acetylation (Gu and Roeder, 1997; Sakaguchi *et al*., 1998) and glycosylation (Shaw *et al*., 1996). Human and/or murine p53 proteins can be phosphorylated by an ever growing number of protein kinases *in vitro*, including the DNA-activated protein kinase (DNA-PK; Lees-Miller *et al*., 1990), the ATM kinase (Banin *et al*., 1998; Canman *et al*., 1998, Khanna *et al*., 1998), the ATR kinase (Canman *et al*., 1998; Tibbetts *et al*., 1999), casein kinase I (Milne *et al*., 1992), the trimeric cdk-activating kinase complex (CAK; Ko *et al*., 1997), c-jun N-terminal kinases (JNKs; Milne *et al*., 1995; Hu *et al*., 1997; Fuchs *et al*., 1998), raf-1 (Jamal and Ziff, 1995) and MAPK (Milne *et al*., 1994) in the N-terminus, and by S- and G_2-phase CDK complexes (CDKs; Wang and Prives, 1995), protein kinase C (Baudier *et al*., 1992), and casein kinase II (Meek *et al*., 1990) in the C-terminus. p53 can also be acetylated *in vitro* at sites within the C-terminus by PCAF and p300 (Gu and Roeder, 1997; Sakaguchi *et al*., 1998). It has yet to be demonstrated which of the above kinases and acetylases actually modify p53 *in vivo*.

Importantly, however, both phosphorylation and acetylation of p53 *in vivo* were shown to be regulated by DNA damage. Phosphorylation at Ser15, -33 and -37 are induced by both UV and ionizing radiation (IR) (Shieh *et al*., 1997; Siliciano *et al*., 1997; Sakaguchi *et al*., 1998). Pertaining to the potential significance of inducible phosphorylation at N-terminal sites is our observation that phosphorylation at Ser15 and -37 disrupts the interaction between p53 and MDM2, and therefore alleviates transcriptional inhibition mediated by MDM2 (Shieh *et al*., 1997). In principle, such interference caused by N-terminal phosphorylation could also inhibit MDM2-targeted p53 degradation and contribute to the stabilization of p53 (reviewed by Prives, 1998).

p53 is also inducibly modified at C-terminal residues. Phosphorylation of Ser392 is induced by UV light but not by IR (Blades and Hupp, 1998; Kapoor and Lozano, 1998; Lu *et al*., 1998). Dephosphorylation is also involved in signalling to p53, since IR triggers dephosphorylation at Ser376, and this facilitates an interaction between p53 and the 14-3-3 protein(s) (Waterman *et al*., 1998). In addition, Sakaguchi *et al*. (1998) have described the induction of acetylation at lysine 382 upon DNA damage, and its regulation by N-terminal phosphorylation.

To elucidate signalling to p53, we have studied further

N-terminal phosphorylation. The kinetics of phosphorylation at N-terminal sites, Ser15 and -33, were examined after UV and IR. In addition, we have identified a new phosphorylation site at Ser20. The fact that Ser20 lies within the MDM2 interaction domain suggests that this modification is likely to be highly significant for regulation of p53 by MDM2. Unexpectedly, we discovered that phosphorylation at these N-terminal sites occurs efficiently *in vivo* only if p53 is an oligomer. The implications of these results are discussed.

Results

Rapid induction of p53 phosphorylation at multiple N-terminal sites upon DNA damage

Three potential amino acid phosphate acceptors, Ser15, -20 and -33, are in close proximity to or within the region on p53 that interacts with MDM2 (residues 17–27). Based on previous results that the interaction of p53 with MDM2 is regulated after DNA damage *in vivo* (Shieh *et al*., 1997), it was of interest to determine the status of phosphorylation at these sites in irradiated and untreated cells. We wished initially to examine whether these three sites are coordinately or differentially regulated after DNA damage. Using previously described procedures (Kitagawa *et al*., 1996), polyclonal antibodies were raised against stably phosphorylated p53 N-terminal peptides spanning these three serine residues. Specificities of the three antibodies were confirmed by ELISA, using phosphorylated and unphosphorylated peptides representing the three respective sites (Obata,T., Tanaka,T., Kumagaye,K., Nakajima,K., Shieh,S.-Y., Prives,C., Tami,K. and Taya,Y., manuscript in preparation). It was previously determined that the αp53-P-Ser15 antibody can be used to detect DNA damage-induced phosphorylation at Ser15 of wild-type p53 (wt p53) in human cells (Shieh *et al*., 1997; Siliciano *et al*, 1997). This was facilitated by treating cells with the calpain/proteasome inhibitor, LLnL, which stabilizes p53 protein (Maki *et al*., 1996) and thus allows comparison of comparable levels of p53 phosphorylated with and without irradiation. We also reported that the αp53-P-Ser33 antibody recognizes p53 in LNCaP and RKO cells after IR, although we could not determine whether Ser33 is constitutively or inducibly phosphorylated in these cells (Ko *et al*., 1997), because treatment of these cells with LLnL causes a marked reduction in reactivity with the αp53-P-Ser33 antibody (data not shown). To facilitate detection of possible induction of phosphorylation at these N-terminal sites, we utilized the human CEM acute lymphoblastic leukemia cell line, which expresses constitutively high levels of p53 protein. While CEM cells' p53 protein is mutated (Cheng and Haas, 1990), they have two advantages for examination of induced phosphorylation after DNA damage. First, they express markedly greater quantities of p53 than in most cells with wt p53, and so it is possible to perform 'straight Westerns' of cell lysates, avoiding possible post-lysis modification of p53 that might occur during immunoprecipitation prior to Western blotting. Secondly, in CEM cells, DNA-damage treatment did not alter the amount of detectable p53 protein, therefore facilitating assessment of the extent of phosphorylation without having to take into account changes in the protein levels (Figure 1A).

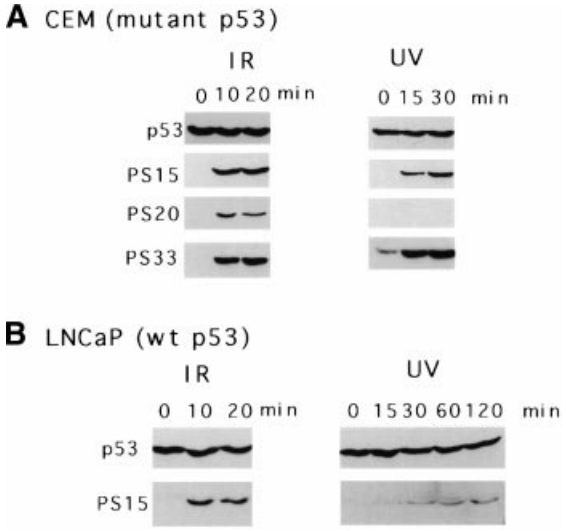

Fig. 1. Rapid induction of phosphorylation at the N-terminus of p53 after DNA damage. (**A**) CEM cells were treated with UV (50 J/m^2) or γ (3.5 Gy) and lysed at indicated time points. Total cell extracts were analyzed by Western blotting using either p53 antibody PAb1801 (p53) or phospho-serine-specific antibodies: αp53-P-Ser15 (PS15), αp53-P-Ser20 (PS20) or αp53-P-Ser33 (PS33) as probes. (**B**) LNCaP cells were treated with the proteasome/calpain inhibitor LLnL (50 μM) for 4 h before being subjected to UV (50 J/m^2) or γ (7 Gy) treatment. Cell lysates were then prepared at the indicated time points and analyzed as described in (A).

After either UV or IR treatment significant induction of phosphorylation, not only at Ser15 but also at Ser33, was observed within 10 min (Figure 1A). Basal phosphorylation was seen at Ser33 under some conditions, whereas it was non-detectable at Ser15 (Figures 1 and 2). In addition to phosphorylation at Ser15 and -33, CEM cell p53 also reacted with the αp53-P-Ser20 antibody rapidly after IR (Figure 1A). Although not induced by UV over this time course, induction of phosphorylation at Ser20 occurs at much later time points (see below). Similar observations were also made with another cell line, HT29, that has mutant p53 (Cottu *et al*., 1996) (Figure 2B).

To exclude the possibility that rapid induction of phosphorylation can only occur in cells that harbor mutant forms of p53, the levels of wt p53 protein in LNCaP human prostate carcinoma cells were increased by treating the cells with LLnL. Cells were first incubated with the proteasome inhibitor for 4 h and then irradiated with either IR or UV. As demonstrated in Figure 1B, phosphorylation at Ser15 was also observed within 10 min of IR in these cells. In contrast to the IR response, however, the UV response in LNCaP cells followed slower kinetics and elicited a lower level of phosphorylation. Under these conditions neither the αp53-P-Ser20 or the αp53-P-Ser33 antibodies reacted with p53 in LNCaP cells. In the former case, the lack of detection may be due to lower sensitivity of the phospho-Ser20-specific antibody. With respect to αp53-P-Ser33, this is probably because, as mentioned above, p53 from LLnL-treated cells is only poorly recognized by this antibody.

To understand further the relationship between these N-terminal phosphorylation events, the kinetics of phosphorylation/dephosphorylation, as determined by reactivity with the three antibodies, were followed in CEM (Figure 2A) and HT29 (Figure 2B) cell lines. Although

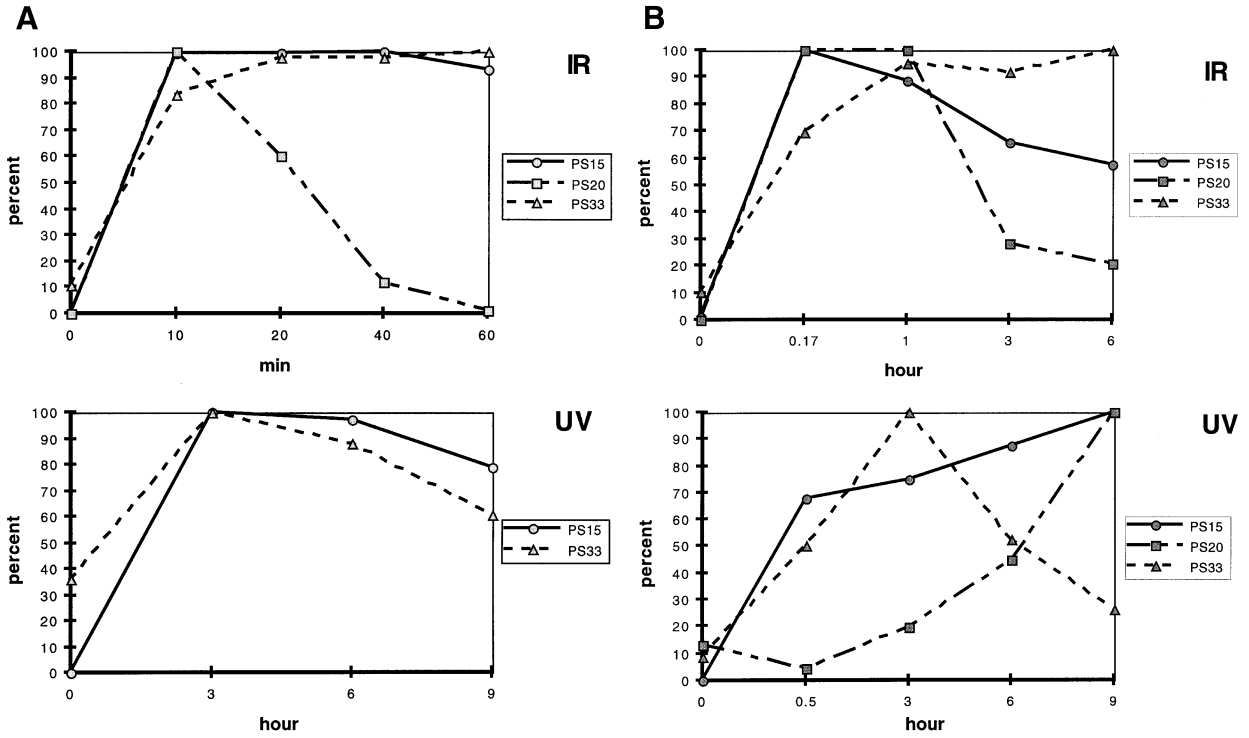

Fig. 2. Kinetics of induction of phosphorylation at Ser15, -20 and -33 of p53 after DNA damage. Whole cell lysates were prepared from CEM (**A**) or HT29 (**B**) cells treated with UV or γ and analyzed as described in Figure 1 legends. The signals detected after Western blotting were quantified using a densitometer (Bio Image, Millipore), and expressed as percentage of phosphorylation relative to the peak value.

the three sites were phosphorylated rapidly after IR, they appeared to be differentially regulated in the two cell types. In CEM cells, phosphorylation at Ser15 and Ser33 was rapid and sustained, while in HT29 cells there was some loss of reactivity with the αp53-P-Ser15 antibody but not with the αp53-P-Ser33 antibody. Moreover, after IR there was a dramatic loss of reactivity with the αp53-P-Ser20 antibody which occurred shortly after the 10 min time point in CEM cells, while in HT29 cells the loss of reactivity to this antibody was somewhat less precipitous. After UV treatment, reactivity with the αp53-P-Ser15 antibody was sustained in both cell types, while reactivity with the αp53-P-Ser33 antibody was slower and more transient in HT29 cells, peaking at 3 h and then falling to near pre-UV levels within 9 h. In contrast, induction of phosphorylation at Ser20 was not observed until 3 h, and remained high at the 9 h time point (Figure 2B). Thus, these three N-terminal sites are inducibly phosphorylated after DNA damage but are differentially regulated with respect to kinetics of phosphorylation and source of DNA damage in a cell-type-specific manner.

DNA damage-induced Ser15 phosphorylation requires the tetramerization but not the DNA-binding regions of p53

An early goal of these experiments was to examine features of the N-terminus that are necessary for phosphorylation at Ser15. We first tested whether the N-terminal domain alone can be phosphorylated *in vivo*. A construct encoding p53 amino acids 1–96 (N96) was introduced into LNCaP cells by transient transfection and the phosphorylation status of the transfected truncated p53 protein was then examined by immunoprecipitation (IP)/Western blotting. Surprisingly, as shown in Figure 3A (lower panel), although the p53 N96 polypeptide was readily detectable in transfected cells, no phosphorylation was apparent on the transfected N-terminal domain, even though phosphorylation of endogenous wt p53 at Ser15 in these cells was evident after UV irradiation (Figure 3A, upper panel).

The last 30 amino acids of p53 have been shown to be required for its interactions with a variety of DNA structures and have been proposed to be a DNA damage recognition domain (Lee *et al*., 1995; Reed *et al*., 1995). Furthermore, phosphorylation of p53 at Ser15 by DNA-PK requires the presence of DNA (Shieh *et al*., 1997 and references therein). It is thus possible that p53 needs to be associated directly with DNA in order to be phosphorylated at this residue. Arguing against the possibility that non-specific interactions of p53 with DNA are required for phosphorylation at Ser15 is the fact that a construct expressing the N-terminal 97 amino acids fused to the C-terminal 30 residues was similarly resistant to phosphorylation at this site (Figure 3A, compare lower and upper panels).

To identify possible signatures in p53 that can direct its N-terminal phosphorylation *in vivo*, additional constructs were generated which express the N-terminal domain fused to different parts of p53. Interestingly, by attaching the full C-terminal domain (amino acids 299–393) to the N-terminus (NC100), the transfected p53 protein construct now became readily phosphorylated at Ser15 (Figure 3B). Thus, sequences within the C-terminus other than the last 30 amino acids are required for N-terminal phosphorylation, suggesting that the tetramerization domain is required for such modification *in vivo*. Indeed, the lack of

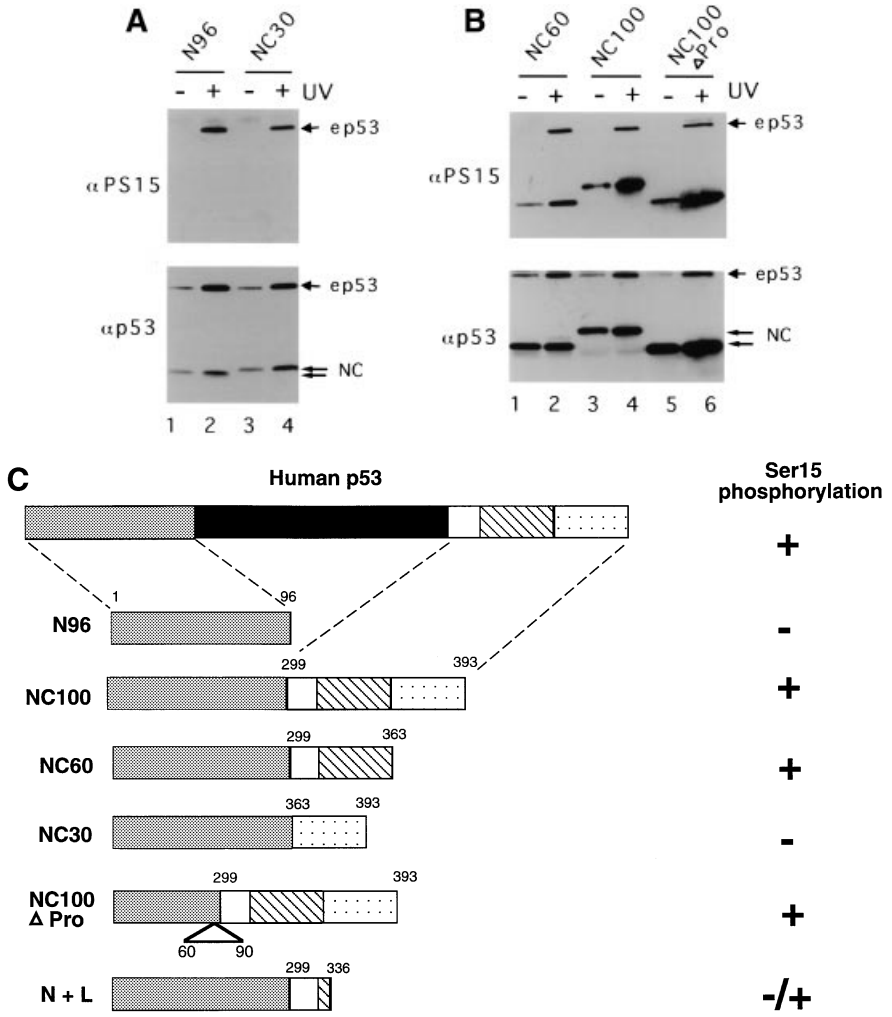

Fig. 3. Efficient phosphorylation of p53 at Ser15 requires the tetramerization domain. (**A** and **B**) LNCaP cells were transiently transfected with plasmids that express either the N-terminal domain alone (N96) or the N-terminal domain plus various regions of the p53 C-terminal domain (NCs). Cell extracts were prepared 40 h after transfection and 8 h after UV treatment, and immunoprecipitated with anti-p53 monoclonal antibody PAb1801 cross-linked to protein A beads. The immunoprecipitates were analyzed by Western blotting using first the αp53-anti-P-Ser15 (αPS15) antibody then the p53 antibody PAb1801 after stripping. 'ep53' indicates endogenous p53. (**C**) Summary of domains required for phosphorylation at Ser15.

requirement for these final 30 residues was confirmed in this background as well, because deletion of that region, generating p53 (NC60) containing the N-terminus fused to the tetramerization region, also supported Ser15 phosphorylation (Figure 3B). In addition, deletion of the PXXP domain (NC100Δpro), a region involved in mediating apoptosis (Walker and Levine, 1996; Sakamuro *et al*., 1997; Venot *et al*., 1998), did not affect Ser15 phosphorylation, since a chimera containing the N-terminal 59 amino acids fused to the C-terminal 94 amino acids was readily phosphorylated at that site (Figure 3B). Figure 3C summarizes the results shown in Figures 3A,B and 4A below. Our data show that both the central core DNA binding domain and the C-terminal basic region are dispensable for directing Ser15 phosphorylation *in vivo*. Thus, upon DNA damage, p53 need not necessarily bind DNA directly in order to be phosphorylated at Ser15.

Phosphorylation of additional sites within the N-terminus requires the tetramerization domain

The results above suggested strongly that the tetramerization domain is critical for efficient phosphorylation at Ser15. Indeed, when a construct lacking the full tetramerization region (N-terminus fused to amino acids 299–336) was generated and transfected into cells, the resulting protein (N+L) became markedly less phosphorylated after IR (Figure 4A). This was even more dramatic when phosphorylation at Ser20 and Ser33 was examined (Figure 4B), in that phosphorylation at these two additional sites was only detected with the NC60 construct containing the tetramerization domain. When cells transfected with these constructs were subjected to UV, essentially identical results were obtained (data not shown). Note that there was strong reactivity with the αp53-P-Ser33 antibody even without IR treatment (Figure 4B). Although the basis for this reactivity is not yet well understood, we have observed that there is frequently more 'basal' phosphorylation at Ser33 than at other sites (Ko *et al*., 1997; unpublished data). It is also possible that since the process of transfection itself can cause a p53 DNA-damage response (Renzing and Lane, 1995), this may trigger only a subset of the p53 signalling pathways, one of which leads to phosphorylation at Ser33.

To rule out differences in cellular localization between

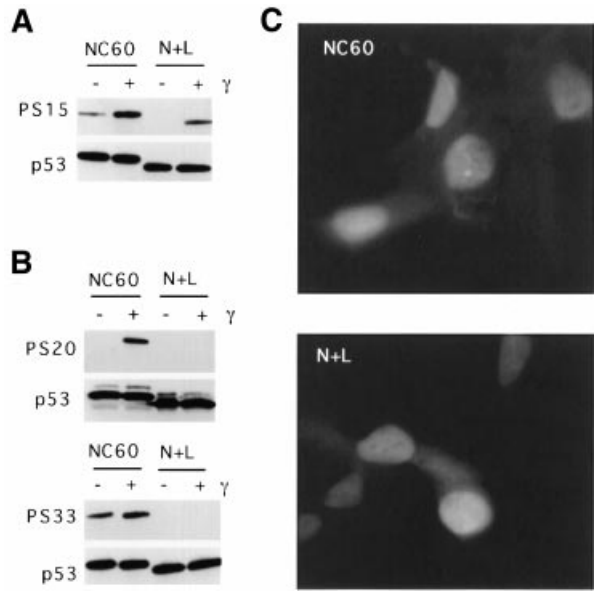

Fig. 4. The tetramerization domain is required for phosphorylation of p53 at Ser20 and -33. LNCaP cells transiently transfected with either N+L or NC60 versions of p53 were irradiated with UV or γ as indicated ~40 h after transfection and cell lysates were prepared 5 h (after UV) or 15 min (after γ) later for immunoprecipitation using hemagglutinin (HA) antibody 12CA5-coupled protein A beads. Phosphorylation of the precipitated proteins was then examined by probing with αp53 anti-P-Ser15 (PS15) antibody (**A**) or αp53 anti-P-Ser20 (PS20) and αp53 anti-P-Ser33 (PS33) antibodies (**B**). The blots were subsequently stripped and re-probed with p53 monoclonal antibodies PAb1801 and DO-1. (**C**) Nuclear localization of the NC60GFP and N+LGFP fusion. LNCaP cells plated on coverslips were transfected with plasmids encoding either NC60GFP or N+LGFP. Cells were fixed 2 days after transfection and mounted for fluorescent microscopy.

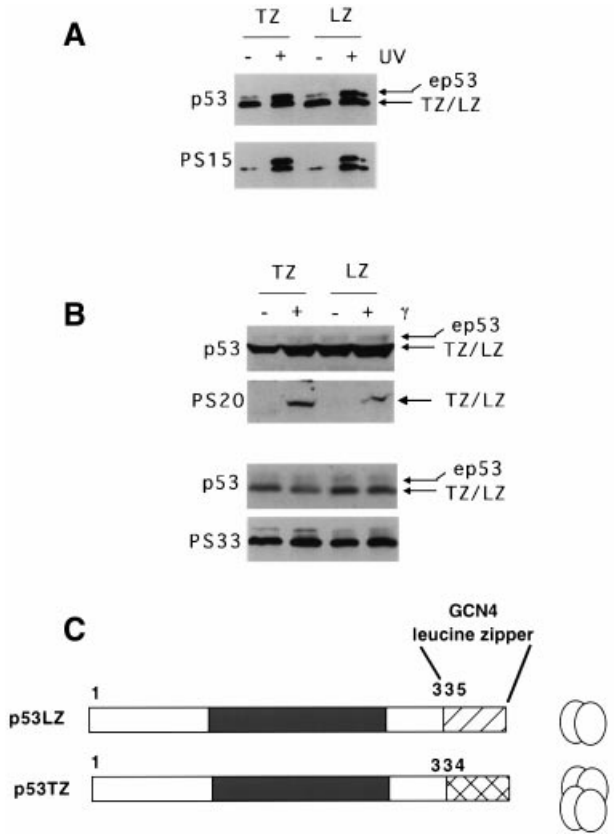

Fig. 5. The tetramerization domain can be replaced by an artificial leucine zipper in directing phosphorylation at Ser15, -20 and -33 *in vivo*. Plasmids encoding either p53TZ, which forms tetramers, or p53LZ, which forms dimers [as illustrated in (**C**)], were transfected into LNCaP cells which were then irradiated with UV (**A**) or γ (**B**) 40 h following transfection. Lysates were prepared either 5 h (for UV) or 15 min (for γ) after the treatment, and processed for immunoprecipitation using PAb1801-coupled protein A–Sepharose beads. Probing was essentially carried out as described in Figure 4. ep53 indicates endogenous p53.

the two forms of p53 containing and lacking the full tetramerization region, these polypeptides were linked through their C-terminal portions to green fluorescent protein (GFP). As shown in Figure 4C, both p53–GFP proteins were detected in the nucleus when transfected into LNCaP cells, with no apparent difference in their localization pattern or levels of expression as evidenced both by IP/Western blotting (Figure 4A and B) and fluorescence microscopy (Figure 4C). We conclude from these results that there is a requirement for the tetramerization domain for efficient phosphorylation at all three N-terminal sites (Ser15, -20 and -33).

The tetramerization domain can be functionally replaced by heterologous oligomerization domains in mediating N-terminal phosphorylation

The results described above suggested that either tetramerization of the p53 protein per se or some other feature(s) of the sequences in this domain is important for N-terminal phosphorylation. To distinguish between these two possibilities, the tetramerization domain of p53 was replaced by either a leucine zipper derived from transcription factor GCN4 which forms dimers (p53335LZ), or a variant of this sequence which forms tetramers (p53334TZ) (Figure 5C). These two chimeric p53 proteins have been shown to form dimers (LZ) and tetramers (TZ), respectively, in gel mobility-shift assays (Waterman *et al*., 1995, 1996). As shown in Figure 5A and B, phosphorylation of both fusion proteins occurred *in vivo* as evidenced by reactivity with all three phospho-serine-specific antibodies. Induction of phosphorylation at Ser15 and Ser20 after DNA damage was evident, while initial levels of phosphorylation at Ser33 were high and additional phosphorylation at that site was not detected. Note that since the TZ/LZ constructs lack the p53 C-terminal 30 amino acid domain, which contains both phosphorylation and acetylation sites shown previously to be inducibly modified after irradiation (reviewed in Giaccia and Kastan, 1998; Prives, 1998), these data further suggest that phosphorylation at the N-terminus of p53 can occur in the absence of C-terminal modifications. Our results demonstrate that N-terminal phosphorylation requires minimal dimerization, and that it is the oligomerization event that mediates DNA damage-induced phosphorylation.

Discussion

Induction of p53 phosphorylation at multiple N-terminal serines

We have demonstrated that there is a concerted induction of p53 phosphorylation at Ser15, -33 and a novel site, Ser20, upon DNA damage. Although phosphorylation at

all three sites can be rapidly induced, they can each be differentially regulated by the type of irradiation and kinetics of phosphorylation/dephosphorylation. Finally, while the DNA binding properties of the p53 protein are apparently unnecessary, phosphorylation of these three sites requires oligomerization of p53.

When considering how cells inducibly phosphorylate p53 after DNA damage, one can envision at least three levels of regulation: (i) the triggering (e.g. IR and UV) events; (ii) the kinases which actually phosphorylate the p53 protein; and (iii) the potential phosphatases involved. It has not yet been established which kinases are responsible either for initiating the signalling cascade or which directly phosphorylate p53 *in vivo*. However, there are a number of suggested candidates derived from *in vitro* studies. With respect to Ser15, three protein kinases have been reported to phosphorylate p53 at this site *in vitro*: DNA-PK (Lees-Miller *et al*., 1990), ATM (Banin *et al*., 1998; Canman *et al*., 1998; Khanna *et al*., 1998) and ATR (Canman *et al*., 1998; Tibbetts *et al*., 1999), all of which are members of the PI-3 kinase family (Lavin and Shiloh, 1997). Although Siliciano *et al.* (1997) reported that there is a significant reduction in the kinetics and extent of phosphorylation in cell lines from AT patients, cells defective in either DNA-PK or ATM are still capable of phosphorylating p53 Ser15 upon DNA damage (unpublished observation). Since p53 does eventually get phosphorylated in cells which lack DNA-PK or ATM, this suggests either that these kinases do not play a direct role in Ser15 phosphorylation *in vivo*, or that there is redundancy such that multiple kinases are involved in signalling to and induction of Ser15 phosphorylation. Less still is known about regulation of Ser33 phosphorylation. At least one kinase, CAK, can phosphorylate p53 at Ser33 *in vitro* (Ko *et al*., 1997), although it is not known whether the kinase is responsible for phosphorylation at this residue *in vivo*. It will be informative to continue to examine how and when p53 is phosphorylated at these three sites using a more extensive range of DNA damaging agents in different cell types. However, determination of the actual kinases required for phosphorylation of p53 N-terminal sites after DNA damage will require a combination of biochemical and genetic approaches.

Our studies have revealed a novel phosphorylation site within p53 at Ser20. Although it is rapidly induced by IR, its induction by UV is significantly delayed when compared with the other two sites. Identification of a Ser20 kinase(s) will probably provide important information about a novel signalling pathway to p53. Ser20 is likely to be important in regulation of p53 protein levels and activity due to the fact that it lies directly within the region required for interaction with MDM2. Indeed, mutation of this residue renders p53 markedly more sensitive to inhibition and destabilization by MDM2, suggesting that phosphorylation of this site *in vivo* reduces its interaction with MDM2 (Unger *et al*., 1999). Our previous experiments suggest that after γ irradiation, p53 interaction with MDM2 is reduced in two cell types when compared to similar levels of p53 from unirradiated cells (Shieh *et al*., 1997). Although we demonstrated that phosphorylation at Ser15 and -37 by DNA-PK reduces the ability of p53 to bind to MDM2 *in vitro*, it is probable that phosphorylation at Ser20 contributes to the reduced interaction of p53 with its negative regulator, and that the ability of p53 to interact with MDM2 is governed by multiple kinases including that which phosphorylates Ser20.

MDM2, however, may not be the only player in regulating p53 stability. In fact, JNK was recently shown to regulate the levels of p53 protein and it was suggested that this stabilization pathway is independent of the ability of MDM2 to affect p53 turnover (Fuchs *et al*., 1998a,b).

Regulation of N-terminal phosphorylation of p53

It is interesting to speculate as to how cells achieve nearly simultaneous induction of phosphorylation at multiple sites, especially when they are very probably mediated by different kinases. One explanation for this coordinated phosphorylation would be that p53 associates with a complex containing multiple N-terminal kinases. Such a complex might exist in a preformed state in cells, or alternatively, it is attractive to further conjecture that the association of diverse kinases may be triggered by DNA damage. The association may be direct, through interaction between kinases and p53; or it can be mediated through certain scaffold proteins, as recently demonstrated for the phosphorylation of IκB (Cohen *et al*., 1998; Scheidereit, 1998) and MAPK (Schaeffer *et al*., 1998; Whitmarsh *et al*., 1998). It would therefore be interesting to know whether the p53 N-terminal kinases form a complex, and if so, how such complexes respond to signals from damaged DNA.

It is not yet understood how or why the tetramerization domain contributes to N-terminal modification events. This domain, the structure of which has been solved by three dimensional NMR (Lee *et al*., 1994; Clore *et al*., 1995) and X-ray crystallography (Jeffrey *et al*., 1995), consists of a pair of dimers. It has been studied previously largely in terms of its influence on the ability of the protein to bind specifically to DNA. While the core domain alone binds well to DNA, there is good evidence that in the context of the intact p53 protein, the tetramerization region contributes to and may be essential for stable high affinity binding of p53 to its cognate site (Waterman *et al*., 1995). Our data suggest that a second function of this domain is to facilitate efficient phosphorylation of sites at the N-terminus of the protein. It is interesting to speculate how this might work. The N-terminus might assume a significantly altered conformation as a monomer, which is less easily recognized by the N-terminal kinases. Juxtaposition of N-termini, mandated by oligomerization of the protein, might somehow affect the shape of this region such that it is more effectively presented to the protein kinases involved in signalling after DNA damage. Alternatively, these protein kinases may require stable interaction with one monomer of a p53 oligomer in order to phosphorylate a neighboring monomer. Indeed, a number of kinases including CAK (Ko *et al*., 1997) and ATM (Khanna *et al*., 1998) have been shown to bind to p53. It is also possible that oligomerization mediates the association indirectly through potential scaffold proteins as mentioned above. Finally, oligomerization may simply increase the local concentration of p53 and thus facilitate its phosphorylation. Since the transfected p53 constructs are overexpressed in cells, the latter possibility seems less likely. Furthermore, p53 N-terminal peptides which do

not oligomerize can be substrates for phosphorylation at Ser15 and -37 *in vitro* (Lees-Miller *et al*., 1992). Whether this reflects non-physiological quantities of proteins and kinases *in vitro* or additional structural and functional requirements for oligomerization *in vivo* remains to be determined. The observation that the ability of MDM2 to bind monomeric p53 is impaired (Marston *et al*., 1995) may be relevant to our observations.

DNA damage recognition in induction of phosphorylation

p53 binds specifically to its target sites through the central core domain (Bargonetti *et al*., 1993; Halazonetis and Kandil, 1993; Pavletich *et al*., 1993; Wang *et al*., 1993). It has also been shown to bind DNA non-specifically through the core domain and the C-terminal basic region (Bakalkin *et al*., 1996; Selivanova *et al*., 1996). It has been speculated that p53 responds to damage signals by binding directly to DNA (Selivanova and Wiman, 1995). However, in our studies we have shown that this need not be the case. By deleting both the core domain and the basic region, p53 can still be phosphorylated upon DNA damage. This is not because the transfected TZ and LZ constructs oligomerize with the endogenous p53, since these chimeras cannot form oligomers with the endogenous p53, yet can still be phosphorylated upon DNA damage. It is more likely that DNA damage signals to p53 through a more indirect way, perhaps through another damage recognition factor(s).

Regulation of p53 function by phosphorylation

Because the N-terminus contains several phosphorylation sites, the relationship between phosphorylation and the transactivation function of p53 has been examined through mutagenic studies. Single mutation of individual N-terminal serines seems to have no or minimal effect on transactivation in transient transfection assays (Fuchs *et al*., 1995), although multiple mutations at specific N-terminal sites poses significant mitigating effects (Mayr *et al*., 1995). Furthermore, Lohrum and Scheidtmann (1996) have demonstrated promoter and cell-type-specific effects of phosphorylation site mutants, suggesting that stress might trigger the onset of different p53 downstream target genes by inducing different combinations of phosphorylation. We observed that p53 phosphorylated by DNA-PK at Ser15 and -37 appears similar to untreated p53 in activating the $p21^{WAF}$ promoter element *in vitro* (Shieh *et al*., 1997). It will be interesting to examine in this way the effect of phosphorylation of additional N-terminal sites on transactivation by p53.

It is possible that other DNA damage-induced events, such as phosphorylation at additional sites, acetylation or even dephosphorylation, may be involved in regulation of p53 transactivation and turnover as well. Consistent with this speculation, induction of phosphorylation, dephosphorylation and acetylation at the C-terminal domain of p53 after DNA damage have been recently demonstrated. It is also quite plausible that through collaboration of the inducible modifications at its N- and C-termini, p53 achieves maximal activation and stabilization.

Materials and methods

Cell lines

HT29, a human colon adenocarcinoma cell line with mutant p53, was maintained in McCoy's 5a medium supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS). CEM, a human acute lymphoblastic leukemia cell line with mutated p53, was grown in RPMI with 2 mM glutamine, 1 mM sodium pyruvate, and 10% FBS. LNCaP cells (human prostate adenocarcinoma cells with wt p53) were maintained in RPMI supplemented with 10% FBS. All three cell lines were obtained from ATCC.

Plasmids

The cDNA encoding the N-terminal 96 amino acids of p53 was generated by PCR with a 3′ primer encoding a HA tag, and cloned into the *Bam*HI and *Eco*RI sites of pCDNA3 (Invitrogen). Various C-terminal fragments were obtained by restriction enzyme digestion and inserted in-frame downstream of N96 in pCDNA3 using *Xho*I and *Xba*I sites. The NC100Δpro construct was made by removing a *Psh*AI–*XCM*I fragment from the NC100.

For visualizing the cellular localization of NC60 and N+L proteins, the *Hin*dIII–*Bpm*I fragment (for NC60) and the *Hin*dIII–*Eco*47III fragment (for N+L) of pCDNANC100 was subcloned into the *Hin*dIII–*Kpn*I sites and the *Hin*dIII–*Sma*I sites of the vector pEGFP-N1 (Clontech), respectively. The resulting plasmids express parts of p53 fused to GFP at the C-termini, and therefore the protein products can be visualized under fluorescent microscope.

For expression of p53TZ/LZ, the cDNAs p53LZ335 and p53TZ334NR (kind gifts of Dr T.Halazonetis) were removed from the pGEM vectors and recloned into pCDNA3 for mammalian expression.

Phospho-serine-specific antibodies

Anti-p53-P-Ser15 and anti-p53-P-Ser33 antibodies have been described previously (Ko *et al*., 1997; Shieh *et al*., 1997). A similar approach was used for generating the anti-p53-P-Ser20 polyclonal antibody; in this case the peptide LSQETFS(PO_3)DLWKLL (amino acids 14–26) was used for immunization of rabbits. These antibodies were subsequently purified through antigen affinity columns. When used in Western blot analyses, antibodies were pre-incubated with their respective unphosphorylated peptide epitopes (1 μg/ml) for 1 h at room temperature to block any residual reactivity with unphosphorylated p53.

DNA damage treatment and cell lysate preparation

Cells were either untreated, treated with UV (50 J/m^2) using a UV cross-linker (Fisher Biotech), or irradiated with γ using a ^{137}Cs source. Dosages of 3.5 Gy were used for treating CEM cells and 7 Gy for other cells. At different time points, cells were collected, washed with PBS, and lysed in buffer containing 10 mM Tris pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol, 0.5% NP-40, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. Cell lysates were then analyzed by Western blot or diluted 2-fold in TEG buffer (10 mM Tris pH 7.5, 1 mM EDTA, 20% glycerol) and analyzed by immunoprecipitation followed by Western blot as described previously (Shieh *et al*., 1997). In the event of transfection, 1 μg of DNA was transfected using the lipofectin reagent (Gibco-BRL) ~36–40 h before DNA-damage treatment.

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